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Functional analysis of the *Agrobacterium tumefaciens* octopine Ti-plasmid left and right T-region border fragments

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Summary

Border fragments of the octopine Ti-plasmid were tested for their ability to restore tumorigenicity of an avirulent mutant carrying a deleted right border. It was found that neither introduction of left border fragments nor that of small right border fragments at the position of the deletion resulted in a complete restoration of oncogenicity. However, insertion of a larger right border fragment in the deletion mutant gave fully oncogenic strains. In the latter case sequences to the right side of the right border repeat were found to be responsible for a complete restoration of oncogenicity. Also a left border repeat inserted together with this enhancer sequence fully restored the oncogenicity of the deletion mutant. The enhancer-sequence on itself was not able to mediate the transfer of the T-region to the plant cell. Border fragments inserted in inverted orientation in the deletion mutant were able to mediate the transfer of the T-region to the plant cell, but at a reduced frequency.

Introduction

Infection of wound sites on dicotyledonous plants with the soil bacterium, *Agrobacterium tumefaciens* results in the development of Crown Gall tumors (38). During infection *Agrobacterium* transfers part of its tumor inducing (Ti) plasmid (40) to the plant cells, which becomes stably integrated into the plant genome (8). This transferred DNA (T-DNA) harbors genes coding for the production of the plant hormones auxin and cytokinin, allowing the tumor cells to grow on hormone free culture media (1, 7, 19, 34, 39). Furthermore T-DNA genes direct the synthesis of amino acid derivatives, called opines. The opines synthesized are characteristic for the type of Ti-plasmid present in *A. tumefaciens* (6, 12). Best studied are octopine and nopaline type Ti-plasmids.

Sequence data showed that the T-region is flanked by two imperfect direct repeats of 24 bp in the Ti-plasmid (3). Studies on the T-DNA content of different transformed plant tumor lines revealed that the integration of T-DNA into the plant genome often takes place at or near these border repeats (17, 24, 36, 37, 44).

Mutagenesis of the T-region showed that none of the T-region genes are essential for the transfer of this DNA to the plant cell (13, 25). The region on the Ti-plasmid, which harbors genes essential for the transfer process is called the virulence (Vir) region (11, 28). This Vir-region itself is not transferred to the plant genome. Products of the Vir-region are probably directly involved in the transfer of the T-region to the plant cell.

The T-region and Vir-region can be separated on different replicons without loss of oncogenicity (9, 14). Even if the T-region is inserted into the chromosome of *A. tumefaciens* and the *vir*-genes are located on a plasmid, the T-region can still be transferred to the plant cell (15). Plant vectors based on such binary systems have been developed for the transfer of foreign DNA to the plant cell (2, 4, 16). The artificial T-region in these vectors embraces only the border fragments containing the 24 bp im-

perfect repeats of the T-region, but is still efficiently transferred to the plant cell if a plasmid with the *vir*-genes is present in the same *Agrobacterium* cell. This proves the importance of the border fragments in the DNA transfer process.

In contrast to the nopaline Ti-plasmid the octopine Ti-plasmid has two separated T-regions (TL- and TR-region), both of which can be found integrated unlinked in transformed plant tissues. Each of these T-regions is flanked by 24 bp imperfect repeats (3). The TL-region harbors the *onc*-genes and the octopine-synthase (*ocs*) gene (29). The TR-region is not essential for tumor induction on the plant.

Studies on the border fragments of the T-region showed that a deletion of the left border repeat did not result in any detectable effect on the T-region transfer process (13, 21), while deletion of the right border of the nopaline T-region (35) and of the octopine T-region (30) resulted in the loss of oncogenicity. The *onc*-genes and the *vir*-genes essential for tumor induction were still intact in these deletion mutants.

Here we report a study aiming to understand why left and right hand border repeats behave differently upon mutation. These differences might be explained either by the difference in orientation of the repeats relative to the *onc*-genes or by a difference in sequence between the 24bp border repeats or differences in their surrounding sequences. A fourth possibility might be the availability of 'pseudo-borders', which are sequences homologous with the 24bp repeats, near some of the deleted border repeats. If available in direct repeat with the remaining border repeat these pseudo-borders might take over the function of the deleted border repeat. To investigate these possibilities we used a right border deletion mutant of the octopine Ti-plasmid to test different border fragments on their ability to complement this mutation.

In the course of our study Peralta *et al.* (32) demonstrated the presence of an enhancer sequence at the right hand side of the right border repeat. Here we report on the interaction of this enhancer sequence with left and right border repeats in the transfer process of the T-region to the plant cell.

Materials and methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1.

DNA isolation procedures

E. coli plasmid DNA was isolated from overnight cultures in LC medium, using the rapid alkaline lysis procedure of Birnboim and Doly (5). Before ligation, desired restriction fragments were isolated from agarose or polyacrylamide gels by electroelution. Total DNA from *A. tumefaciens* was prepared

Table 1. Bacterial strains used in this study.

Strain	Markers	Plasmid	Reference or source
A. tumefaciens			
LBA1010	rif	pTiB6	(23)
LBA288	rif	-	(18)
LBA4417	rif pTi (Sm Ocs- Ape- Occ-)	pAL4417	(30)
E. coli			
KMBL1164	thi pro lac	-	P. van de Putte
JM101	(lac proAB) supE thi F′ (D36 proAB lacI^{Q}z M15)	-	(43)
GM48	dam3 dcm6 thr1 leu6 thi1 lacY1 galK2 galT22 ara14 tonA31 tsx78 supE44	-	C. C. Richardson
Ce140	Km Tra^{+} (RK2)	pRK2013	(10)

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according to Ooms *et al.* (29).

Bacterial conjugation

pBR322 derivatives were transferred from *E. coli* KMBL1164 to *A. tumefaciens* LBA4417 by triparental matings using pRK2013 as the helper plasmid (18).

DNA manipulations

Restriction-endonuclease digestion, dephosphorylation, agarose-gel electrophoresis, Southern blotting, DNA-DNA filter hybridization, nick translation and autoradiography were performed essentially as described in Maniatis *et al.* (26).

Border fragment clones

pRAL5001 contains the TL right border repeat on a 315 bp BamHI-SstI restriction fragment, coordinates 13774 to 14098 (3), inserted into the pIC19R multilinker (27).

pRAL5002 contains the TR left border repeat on a 965 bp BclI restriction fragment (coordinates 14973 to 15938) inserted into the BamHI site of pIC19R, with the pIC19R XhoI site on the right hand side of the border fragment.

pRAL5003 contains a 622 bp SstI-BclI fragment (coordinates 14089 to 14711) inserted into the SstI and BamHI sites of pIC19R. pRAL5004 contains the TL right border repeat on a 937 bp BamHI-BclI restriction fragment (coordinates 13774 to 14711) inserted into the BamHI site of pIC19R.

To insert the right hand side flanking region of the TL right border repeat next to the TR left border repeat, this region was excised from pRAL5003 as a SmaI-XhoI (pIC19R sites) fragment and ligated into EcoRV-SalI cut pRAL5002. The resulting plasmid, pRAL5005, contains both the TR left border repeat and the TL right border flanking region in wild type orientation.

Introduction of border fragments in LBA4417

Border fragments to be assayed for restoration of oncogenicity in LBA4417 were cloned into the EcoRI site of pRAL5200. This plasmid is a pBR322 derivative, containing a 3761 bp BamHI-EcoRI restriction fragment from the pTiAch5 BamHI-17A fragment, exhibiting overlap and allowing homologous recombination with the right hand end of T-DNA in LBA4417. Furthermore, pRAL5200 contains the spectinomycin resistance marker from Tn7 as a 3 Kb BamHI restriction fragment, inserted into the pBR322 BamHI site. Introduction of an extra

Table 2. Oncogenicity assays of *A. tumefaciens* strains harboring cointegrate plasmids resulting from homologous recombination between pAL4417 and derivatives of the shuttle vector pRAL5200 containing different T-region border fragments. Every strain was tested at least in duplicate on each plant species. Abbreviations used for the description of border fragments are explained in the text. Plants were scored 3 weeks after inoculation.

Strain	Border fragment	Oncogenicity assays*				
		K. daigr	*K. tubi*	*N. glauca*	*L. esc*	*P. sat*
LBA288		−	−	−	−	−
LBA1010 (pTiB6)		+ + +	+ + +	+ + +	+ + +	+ + +
LBA4417 (pAL4417)		−	−	−	−	−
LBA5200 (pAL5200)	− (shuttle vector)	−	−	−	−	−
LBA5201 (pAL5201)	LB	+	+	+	+	+/−
LBA5202 (pAL5202)	LB inverted	−	+/−	−	−	−
LBA5203 (pAL5203)	RB	+	+	+	+	+/−
LBA5204 (pAL5204)	RB inverted	−	+/−	−	−	−
LBA5205 (pAL5205)	LBE	+ + +	+ + +	+ + +	+ + +	+ + +
LBA5206 (pAL5206)	LBE inverted	+ +	+ +	+ +	+/−	+/−
LBA5207 (pAL5207)	RBE	+ + +	+ + +	+ + +	+ + +	+ + +
LBA5208 (pAL5208)	RBE inverted	+	+	+	+/−	+/−
LBA5209 (pAL5209)	E	−	−	−	−	−
LBA5210 (pAL5210)	E inverted	−	−	−	−	−

*: Symbols indicate: −, avirulent; +/−, very weakly virulent; + to + +, partially virulent; + + +, fully virulent.

marker in addition to the pBR322 carbenicillin resistance marker is necessary to allow efficient selection of exconjugants in an *A. tumefaciens* Ach5 chromosomal background.

A list of the pRAL5200 derived plasmids is presented in Table 2. After triparental mating with pRK2013 as a helper plasmid, exconjugants were selected by plating on mineral medium with glucose (41), supplemented with the antibiotics rifampicin (20 μg/ml) and spectinomycin (250 μg/ml).

The structure of all exconjugants was checked by hybridization analysis.

Virulence assays

Two independent exconjugants of every triparental mating were each tested in duplo on stems of *Nicotiana glauca, Kalanchoe daigremontiana, K. tubiflora* and *Lycopersicon esculentum* as described previously (28).

Furthermore, virulence assays were performed on decapitated seedlings of *Pisum sativum.*

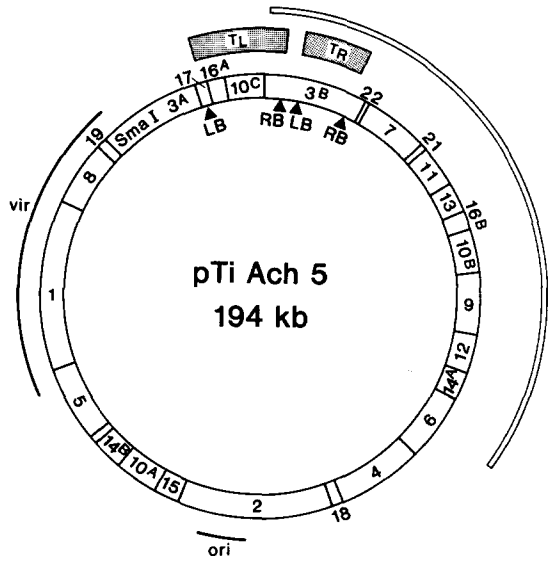

Fig. 1. SmaI restriction map of the octopine Ti plasmid pTiAch5 (30) (TL; TR: TL and TR region, respectively). Triangle indicates positions of left (LB) and right (RB) 24 bp border repeats. Open bar indicates the deletion in pAL4417.

Results

A system for the assay of border activities

In the experiments described below, we used a pTiAch5 right border mutant called LBA4417. This mutant, which was isolated in our laboratory previously (30), harbors an octopine Ti-plasmid (pAL4417) with a 60 Kb deletion from within fragment SmaI-6 up to a site within fragment BamHI-17a in the TL-region (Fig. 1). Therefore, pAL4417 lacks the genes for octopine catabolism (*occ*) and phage AP-1 exclusion (*ape*). We performed a detailed restriction enzyme analysis to map the endpoint of the deletion within the TL-region as accurately as possible. This revealed that the deletion ends in the coding sequence of the octopine synthase gene between base-coordinates 11205 and 11832 on the map of Barker *et al.,* 1983 (3). This means that in pAL4417 the TR-region is completely deleted as well as the right border repeat of the TL-region, but all the *onc* and *vir* genes, which are necessary for plant tumor induction, are still present on the plasmid.

Inoculation of LBA4417 on *Kalanchoe daigremontiana* did not result in tumor formation. Eight weeks after infection no tumors were visible, whereas large tumors were present already three weeks after inoculation with a wild-type strain like LBA1010 (Table 2).

In order to prove that the loss of oncogenicity of LBA4417 is due to the deletion of its right border repeat indeed, we developed a shuttle vector which enabled us to reintroduce the border repeat in pAL4417. This shuttle vector, called pRAL5200, consists of pBR322, a spectinomycin resistance (Sp^r) marker and a pTiAch5 fragment (Fig. 2). This latter pTiAch5-DNA fragment provides a homologous region of approximately 2.1 Kb between the shuttle plasmid and pAL4417. After introduction of the shuttle vector in LBA4417 recombination can occur between the 2.1 Kb segment and its homologous counterpart, which is situated adjacent to the left end of the deletion in pAL4417. This results in the integration of the complete shuttle vector in pAL4417 at the right hand side of the *onc*-genes (see Materials and Methods). In the shuttle vector the unique EcoRI-restriction site present adjacent to this homologous region, was used for the insertion of different border fragments. After integration of the (loaded) shuttle vector into pAL4417 the load (border fragments) ends up

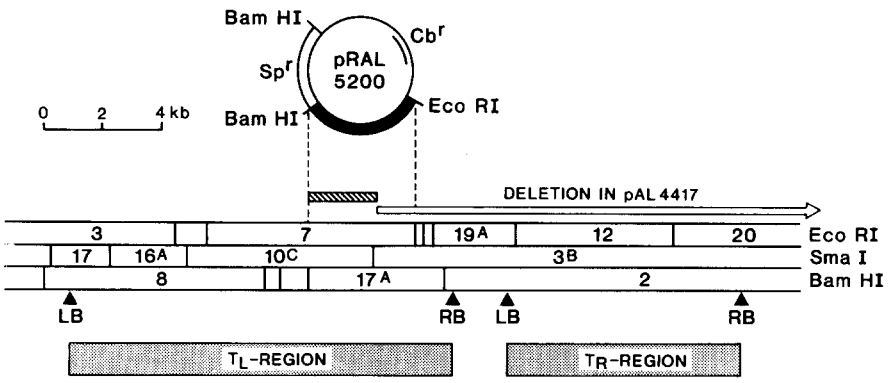

Fig. 2. EcoRI, SmaI and BamHI restriction map of the octopine T-region of pTiAch5. Open bar represents the deletion in pAL4417, hatched bar indicates the homologous region between the shuttle vector pRAL5200 and pAL4417.

directly at the place of the deleted right border (Fig. 2).

Using the shuttle vector the deleted right border fragment, called *RBE,* was reintroduced into pAL4417. This resulted in a complete restoration of the oncogenicity of LBA4417 (Table 2; strain LBA5207). Therefore, we conclude that the loss of oncogenicity of LBA4417 indeed is due solely to the deletion of the right border fragment of its TL-region.

In the following parts of this paper we describe experiments in which we used the shuttle vector to introduce different border fragments into pAL4417. In all these experiments we verified by Southern blotting experiments whether the shuttle vector with the border fragments had inserted into pAL4417 at the proper position and in the correct orientation (Fig. 3A). Since it was possible that more than one copy of the (loaded) shuttle vector had integrated into pAL4417, we also checked the number of copies present in the different constructs by Southern blotting.

It appeared that in most cases only one copy of the shuttle vector has integrated into pAL4417. For comparative biological assays we only used strains with constructs that contained the (loaded) shuttle vector as a single insert (Fig. 3B). However, the number of inserted copies present did not have a drastic influence on the oncogenicity of the strains.

Left and right border regions play a different role in the T-region transfer process

In contrast with the strain containing a wild-type Ti plasmid, LBA1010, which induced large tumors on all plants tested, LBA4417 did not induce any

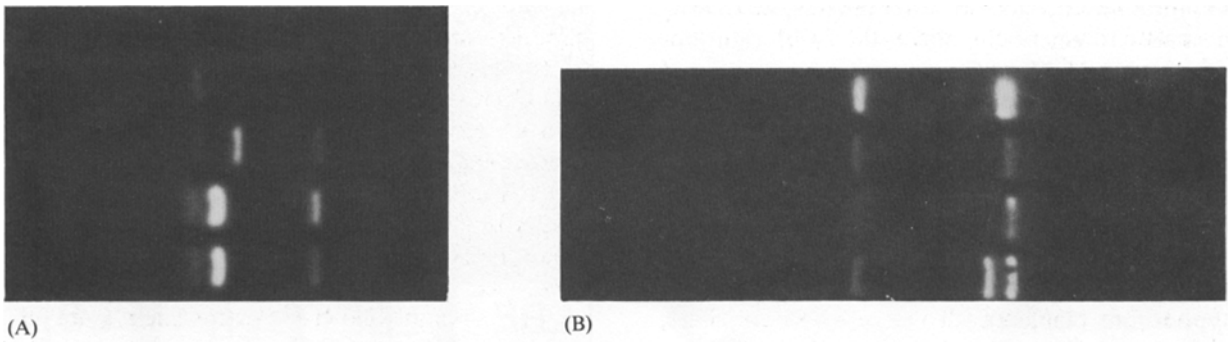

Fig. 3. (A) Southern blots of total DNA isolations from strains LBA4417, LBA5200, LBA5205 and LBA5206 digested with BamHI and probed with pRAL5200. (B) Southern blots of total DNA from independent exconjugants from triparental matings with pRAL5205 and pRAL5206 digested with EcoRI and probed with pBR322 containing the EcoRI-19a fragment of pTiAch5. Lane 1–3 show single inserts of the shuttle vector, lane 4 shows a multiple insertion.

visible tumor on *K. tubiflora, K. daigremontiana, P. sativum, N. glauca* and *L. esculentum* within 8 weeks after inoculation.

Infection of plants with a derivative of LBA4417, containing the borderless shuttle vector pRAL5200 in pAL4417, gave exactly the same negative response as LBA4417 (Table 2; strain LBA5200). Thus the sequences present in pRAL5200 cannot restore oncogenicity in LBA4417.

With the objective to test whether the orientation of a border repeat is of importance in the transfer of the T-region to the plant cell, we first introduced the right border fragment (*RBE*) of the octopine TL-region in pAL4417, in an inverted orientation – relative to the left border repeat – in pAL4417. The resulting strain (LBA5208) was definitely more tumorigenic than LBA4417, but less than wild-type strains (Table 2). This shows that the orientation of this right border fragment relative to the *onc*-genes is important in the transfer process. Insertion of the left border fragment of the octopine TR-region (*LB*) in pAL4417 in either orientation resulted in strains that gave very attenuated responses on plants after inoculation (Table 2; strains LBA5201 and LBA5202). This showed that apart from the difference in orientation between left and right border repeat relative to the *onc*-genes in the T-region there is another functional difference between left and right border fragments.

Sequences adjacent to the right border repeat enhance the transfer of DNA to the plant cell

To study whether sequences surrounding the right border repeat of the octopine TL-region are important in the DNA-transfer process, we inserted a smaller fragment containing the 24 bp right border repeat in pAL4417.

Introduction in pAL4417 of a 315 bp (BamHI-SstI) fragment, called *RB*, of the octopine Ti-plasmid into pAL4417 resulted in strain LBA5203. Fragment RB harbors the right 24 bp border repeat of the TL-region flanked by only 7 bp of Ti-DNA on its right-hand side. Strain LBA5203 induced tumors but was clearly less oncogenic than wild-type strains (Table 2).

As described above insertion of a larger fragment containing besides the right border repeat of the TL-region an extra 622 bp to the right of the 24 bp repeat into pAL4417, resulted in strains such as LBA5207 that are able to induce tumors of a size comparable to those induced by wild-types.

It can therefore be concluded that apart from the right border repeat also a sequence to the right of this repeat is important for tumor induction. This second sequence is apparently not absolutely essential for T-region transfer, but functions as an enhancer-like sequence.

Effect of the enhancer-like sequence on different border repeats

Strain LBA5201, which contains pAL4417 with the left border repeat of the octopine TR-region (*LB*) inserted in direct repeat with the TL-region left border repeat gave a tumor response comparable with a strain containing the right border repeat without the enhancing sequence (LBA5203). However, when the 622 bp SstI-BclI fragment (*E*) containing the enhancer-like sequence of the right border was cloned to the right of this left border repeat in its native orientation (*LBE*), the resulting strain (LBA5205) induced tumors of a wild-type size on the plants tested (Fig. 4; Table 2).

Insertion of this enhancing fragment (*E*) in either orientation in pAL4417 in the absence of a border repeat (LBA5209 and LBA5210) did not result in a tumorigenic strain. This underlines the prime importance of the 24 bp imperfect repeats in the process of T-region transfer to the plant.

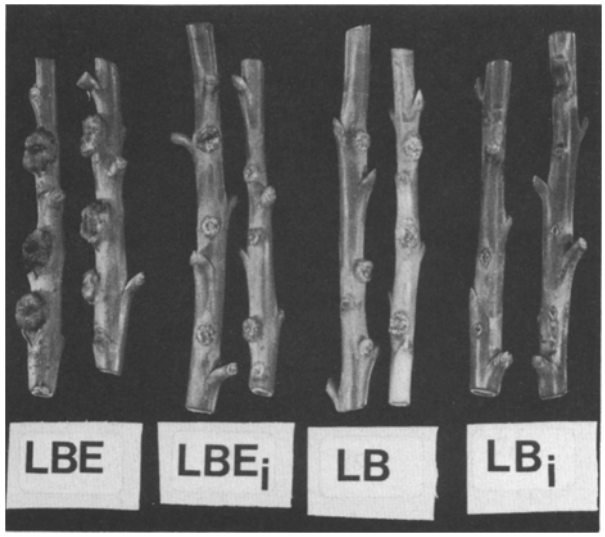

Fig. 4. Stem segments of *N. glauca* three weeks after inoculation with strains LBA5205, LBA5206, LBA5201 and LBA5202.

When introduced in inverted orientation – compared to the left border repeat in pAL4417 – both the left (*LB*) and the right border repeat (*RB*) gave rise to strains (LBA5202 and LBA5204) that induced no or very attenuated tumors dependent on the plant species tested. The presence of the 622 bp enhancing fragment next to these inverted border repeats (*LBE* and *RBE*) resulted in strains (LBA5206 and LBA5208), that were much more oncogenic than LBA5202 and LBA5204 (Table 2). Strain LBA5206, containing the left border repeat in inverted orientation was more oncogenic than LBA5208 with the right border fragment in inverted orientation. In these cases the enhancer-like sequence is present on the left hand side of the border repeat and situated in inverted orientation relative to the *onc*-genes. In spite of an inverted orientation the enhancer fragment apparently still has the capacity to stimulate the frequency of T-region transfer.

Discussion

For our studies we used a system developed previously for the nopaline Ti-plasmid (42), to study the function of border fragments in an octopine Ti-plasmid. For this purpose we used a non-oncogenic Ti-plasmid mutant (pAL4417) deleted over its right TL-region border repeat (30). Via a shuttle vector called pRAL5200 we were able to reinsert, by a single cross over event, border fragments into pAL4417.

Insertion of the shuttle vector itself in pAL4417 did not make the host oncogenic. This shows that the vector does not contain any sequences that can promote transfer of the *onc*-genes to the plant cell. However reinsertion of a right border fragment in pAL4417 (via the shuttle vector) restored the oncogenicity of the strain to wild-type level (LBA5207). Therefore we concluded that the loss of oncogenicity of LBA4417 is due to the deletion of its right border fragment indeed.

The fact that LBA4417 lost its oncogenicity due to the deletion of the right border fragment means that the left border, which is still available in pAL4417 is not able to mediate the transfer of *onc*-genes to the plant cell on its own. In contrast mutants that lack the left border repeat can still efficiently transfer the *onc*-genes to the plant cell (13, 21).

The difference between left border and right border deletion mutants in their ability to transfer the *onc*-genes to the plant cell can be explained in various ways; 1) by a difference in orientation of the border repeat relative to the *onc*-genes, 2) by differences in the sequences of the 24 bp border repeats, 3) by the presence of pseudo border repeats near the deleted border repeat, 4) by differences in the surrounding sequences of the 24 bp border repeats. Our results show clearly that a right border repeat together with surrounding sequences, not only in normal, but also in inverted orientation can mediate the transfer of the T-region to the plant cell. We therefore conclude that it is not the difference in orientation that is responsible for the large difference in effect between left and right border repeats.

We subsequently sorted out whether the difference between a left and right border repeat was due mainly to differences between the 24 bp border repeats themselves or to their surrounding sequences. Our results show that a 622 bp fragment on the right hand side of the right 24 bp border repeat of the octopine TL-region is responsible for enhancing the transfer from right border repeats. Peralta *et al.* found a similar effect and suggested that this enhancer is represented by a consensus sequence, called 'overdrive', found next to the right border repeats of the octopine TL-region and TR-region (32).

In the experiments presented here it was shown that the enhancer fragment is not only able to enhance the T-region transfer mediated by a right border repeat but also (and as efficiently) the DNA transfer mediated by a left border repeat. Thus the enhancing sequence works also in combination with left border repeats. The fact that a left border repeat can function efficiently as a right border repeat in transferring the T-region to the plant proves that left and right border 24 bp sequences can perform identical functions.

The observation made in our experiments that a left border repeat – when inserted on the place of the right border repeat – can transfer the T-region to the plant cell as efficiently as the original right border repeat is in contrast with a report of Rubin (33). Using a small (14Kb) binary construct he found that the left border repeat of the octopine TL-region is not able to transfer *onc*-genes to the plant cell. The difference between the experiments done by Rubin (33) and ourselves is that Rubin used a large fragment containing the left border of

the octopine TL-region, while we used a fragment (*LB*) containing the left border of the TR-region with only a few bases (20 bp) of Ti-DNA on the right hand side of the 24 bp repeat. Therefore it cannot be excluded that sequences surrounding the left border repeats might have an inhibiting function in the T-region transfer process. This possibility is currently under investigation in our laboratory.

Results presented here with the octopine right border repeat are in disagreement with the results obtained by Wang *et al.* (42) who have studied the right border repeat of the nopaline T-region. They reported that a synthetic right border repeat (25 bp) of the nopaline T-region is able to mediate DNA-transfer with wild-type efficiency (42). However from the experiments presented here it is shown that the transfer of the octopine T-region mediated by the right and left border repeat in the absence of an enhancing sequence occurs with a significantly lower efficiency.

These results suggest that certain elements in the transfer mechanism used to transfer a nopaline T-region are somehow different from the mechanism used to transfer an octopine T-region. One of such differences could lie in the absence of a functional enhancer-like sequence in the nopaline Ti-plasmid. Another possibility is that in the constructions used by Wang *et al.* (42) an unidentified enhancer-like sequence is available coincidentally near the insertion site of the border repeats. Currently we are testing the nopaline border repeats in our system to investigate these possibilities. Furthermore we will use this system to test synthetic 24 bp border repeats as well as mutated derivatives in order to determine the nucleotides essential for border activity.

Our experiments in which border fragments with or without the enhancer were inserted in inverted repeat relative to the left border repeat in pAL4417, have shown that these border fragments were still able to mediate the transfer of *onc*-genes to the plant cell. Without the enhancer, however the tumorous response was visible only on certain plant species. With the enhancer fragment (*E*) present next to the border repeats in inverted orientation the tumorous response was strongly increased. This demonstrated the general enhancer like character of the enhancer fragment. Apparently this sequence can still stimulate the efficiency of T-region transfer, even when present on the left side of the border repeat and in an opposite orientation relative to the *onc*-genes. Similar characteristics have been found for enhancer sequences of the G-inversion system of bacteriophage Mu (22) and of the Hin-mediated site-specific recombination in *Salmonella* (20).

We unexpectedly found that a fragment containing the left border repeat with the enhancer-like sequence (*LBE*), together in an inverted orientation, mediates T-region transfer more efficiently than a fragment containing the right border repeat with its enhancer (*RBE*) together in an inverted orientation (LBA5206 and LBA5208). If the 24 bp repeats would be functional in inverted orientation an identical response would have been expected. In order to explain these results we might assume that other sequences ('pseudo borders') present in the border fragments – in direct repeat relative to the left border repeat – are mediating transfer instead of the regular 24 bp sequences. By comparison of the DNA sequences of the fragments inserted in pAL4417 we found a more conserved pseudo border in the fragment containing the left border (TGGCAGAAGCAGGTATCGAGCC) than in the right border fragment (GACTGGCAGGATATATACCGTT). This might account for the detected difference in oncogenicity between LBA5206 and LBA5208.

In order to obtain an answer to the question which border repeat is used for T-region transfer in these cases, we will investigate the T-DNA contents of the transformed plant tissues obtained.

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