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Volume 16 Number 21 1988 Nucleic Acids Research

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Received July 4, 1988; Revised and Accepted September 30, 1988

ABSTRACT

T-region transfer from wild-type Agrobacterium strains is thought to be an orientated process, starting at the right border repeat and terminating at the left border repeat of the T-region. Here we demonstrate that a right border repeat in the inverted orientation relative to the onc-genes can also mediate transfer of the T-region to the plant cell, although with lower efficiency as a border repeat in the native orientation. Transfer mediated by an inverted right border repeat is stimulated by the presence of the T-region transfer enhancer. Similar single stranded molecules, comprising the bottom strand of the T-DNA, were isolated from acetosyringone induced bacteria, irrespective of the orientation of the right border. These findings show that border repeats work bidirectionally to some extent.

INTRODUCTION

Induction of the plant disease Crown Gall by Agrobacterium tumefaciens is the result of DNA-transfer from the bacterium to the plant cell. The transferred DNA (T-DNA) is part of a large extrachromosomal replicon - called the tumor inducing (Ti) plasmid - which is present in the bacterium. After transfer to the plant cell the T-DNA is inserted into the plant genome and expressed into several proteins. Some of these catalyze the production of phytohormones of the auxin and cytokinin classes. This leads to a disturbed hormone balance in transformed plant cells, which as a consequence start growing. Thus, eventually, tumorous overgrowths are formed (1-5). Other T-DNA encoded proteins specify the production of opines, which are amino acid derivatives that are characteristic for the transformed plant cells (6,7).

Apart from the T-region a large virulence (Vir) region is present on the Ti-plasmid. Seven operons (called virA to virG) which together contain more than twenty genes determine a system for the transfer of the T-region to the plant cell (8,9). The functions determined by some of the vir-genes are known (10,11). One of the early steps in the T-region transfer process is the specific nicking of 24bp imperfect direct repeats, which are present at the

boundaries of the T-region, by proteins determined by the virD operon (12-14). Hereafter, single stranded DNA molecules (T-strands) are formed in the bacterium, representing the bottom strand of the T-region (15,16). Deletion of the right border repeat of the T-region renders Agrobacterium avirulent, while deletion of the left border repeat does not affect tumorigenicity (17-21). Therefore processing of the T-DNA in the bacterium is thought to be an orientated process starting at the nick site in the right border repeat. Next to the right border repeat a T-region transfer enhancer (24 bp) is present, which can stimulate T-strand formation not only from its normal position, which is close to the right border repeat, but also in an orientation-independent manner from a distance (of more than 4000 bp) from this border repeat (22,23).

The assumption that T-region transfer is an orientated process is in line with the observation that a reversion of the orientation of the right border repeat relative to the T-region leads to avirulence of the host bacterium (21,24,25). We investigated T-strand formation in strains with normal and inverted right border repeats, and discuss our results in relation to current models for T-DNA transfer.

MATERIALS AND METHODS

Bacterial strains

For cloning experiments we used the Escherichia coli strains JM101 (lac, proAB, supE, thi, F'traD36, proAB, lacIQ, lacZΔM15; 26) and KMBL1164 (thi, pro, lac; P. van de Putte, Leiden).

In the conjugation experiments we used the E.coli strains KMBL1164 and HB101 (pRK2013) (27) and the A.tumefaciens strain LBA4417 (Rif, pTiAch5, Sm, ocs, ape, occ, onc; 19).

A.tumefaciens strains LBA1010 (Rif; pTiB6) and LBA288 (Rif; no Ti-plasmid) were used as a positive and negative control in the virulence assays (28,29).

Bacterial plasmids

The helper plasmid pRK2013 was used for the mobilization of non-conjugative plasmids (27). pIC-vectors 19R; 19H; 20R and 20H were used as initial cloning vectors (30). Border fragments to be introduced into pAL4417 were cloned into the EcoRI site of the shuttle vector pRAL5200 (24).

DNA isolation procedures

Plasmid DNA was isolated from E.coli strains as described by Birnboim and Doly (31) and total DNA from A.tumefaciens as described by Ooms et al. (32).

Construction of plasmids

For cloning experiments the protocols described in the laboratory manual of Maniatis et al. were used (33).

The synthetic right border repeat (SRB) was cloned into the pIC-vectors as a SaII-HindIII fragment. The sequence used was:GTCGACCCCGGGCAGGATATATACCGTTG-TAATTAAGCTT. The enhancer fragment was cloned from the octopine Ti-plasmid pTiAch5 as a 189bp SstI-NruI fragment - coordinates 14087-14276 on the map of Barker et al. (34) - into the pIC-vectors.

Border fragments were initially cloned into pIC19R or pIC20R vectors, and subsequently inserted as EcoRI-fragments into the unique EcoRI-site of pRAL5200 in both orientations. By using primers complementary to the DNA on both sides of this EcoRI-site, the inserted fragments were checked by supercoiled sequencing (35).

Conjugation experiments

The "loaded" shuttle vector was mobilized to LBA4417 in a triparental mating with the helper plasmid pRK2013 (27). Tranconjugants with the (loaded) shuttle vector present in pAL4417 by a single cross-over event (homology of approximately 2.1kbp), were selected for on mineral medium plates (36) containing the antibiotics rifampicin (20ug/ml) and spectinomycin (250ug/ml).

Position, orientation and copy number of the shuttle insert in pAL4417 were checked using Southern blots of BamHI, EcoRI, and if necessary EcoRV digests of total DNAs (37).

Isolation of single stranded T-molecules

Bacteria were grown in mineral medium (pH=5.3) and induced by acetosyringone for 18 hours (15). Total DNA was isolated from these bacteria as described by Ooms et al. (32), and loaded undigested on 0.6% Tris Borate EDTA agarose gels. After electrophoresis the DNA was transferred to nitrocellulose membranes under non-denaturing conditions by Southern blotting. The membrane was hybridized with a T-DNA specific probe (pIC19H-RsaI fragment, containing transcript 4 of the T-region of pTiAch5, coordinates 8497-9836). Strand specific probes were made after insertion of the RsaI-fragment into the SmaI-site of the M13mp8/9 vectors. Subsequently, single stranded DNA was isolated from the phages and this was used as a template for the production of single stranded probes. Strand specific probes against the spectinomycin resistance marker of the shuttle vector were made by primer extension reactions from primers located on either side of the spectinomycin resistance gene, present in pIC19R. T7 RNA polymerase was used

to isolate single stranded RNA probes from inserts cloned in a bluescript vector.

Virulence assays

Inoculation of the constructed strains on test plants was performed as described before (38). For each experiment we used two independent transconjugants from each cross and tested them both at least twice on four different test plants. The test plants used in this study were Nicotiana glauca, Kalanchoe daigremontiana, Kalanchoe tubiflora and Lycopersicon esculentum. All plants were scored three weeks after inoculation, unless stated otherwise.

RESULTS

T-region transfer to the plant cell mediated by an inverted 24bp border repeat.

Border repeats were tested for their ability to mediate T-region transfer after introduction into the Ti-plasmid deletion mutant LBA4417. This deletion mutant lacks the entire TR-region and the right border repeat of the TL-region of its Ti-plasmid, and therefore is non-oncogenic, in spite of the presence of all onc- and vir-genes on this plasmid (24). We reintroduced the 24bp right border repeat into pAL4417 both in the native (LBA5251) and in the inverted orientation (LBA5252) relative to the left border repeat and tested the resulting strains for their ability to induce tumorous growths on several plant species. As can be seen in table I strain LBA5252 provoked little or no tumour response on the test plants three weeks after inoculation, and was clearly less virulent than LBA5251. However, when the incubation time was extended to six weeks tumours became visible on the more sensitive plant species (Nicotiana glauca and Kalanchoe tubiflora). In the same period of time no response at all was observed on plants inoculated with the parental strain LBA4417, which is lacking the right border repeat. These data indicate that an inverted border repeat is able to mediate T-region transfer to the plant cell, albeit with a much lower efficiency than a border repeat in the wild-type orientation (LBA5251).

The fact that tumours induced by LBA5252 as well as LBA5251 develop slowly is at least partially due to the absence of the enhancer sequence called overdrive (22), in the constructs used in these experiments. The overdrive sequence stimulates T-region transfer mediated by a border repeat, but cannot mediate transfer by itself. Therefore, insertion of overdrive alone into pAL4417 in either orientation, does not lead to an increased

Table I. Oncogenicity assays.

Strain	Border fragment	Oncogenicity assays			
		L.esc	K.dai	K.tubi	N.glauca
LBA1010(pTiB6)		+++	+++	+++	+++
LBA4417(pAL4417)		-	-	-	-
LBA5200(pAL5200)	- (shuttle vector)	-	-	-	-
LBA5235(pAL5235)	N	-	-	-	-
LBA5236(pAL5236)	N-inverted	-	-	-	-
LBA5251(pAL5251)	SRB	+/-	+	+	+
LBA5252(pAL5252)	SRB-inverted	-	-	+/-	+/-
LBA5265(pAL5265)	SRBN	+++	+++	+++	+++
LBA5266(pAL5266)	SRBN-inverted	+/-	+	++	++
LBA5269(pAL5269)	NSRB	+++	+++	+++	+++
LBA5270(pAL5270)	NSRB-inverted	+/-	+	++	++

Plants were scored 3 weeks after inoculation. Each strain was tested at least twice on each of the plant species.
Abbreviations: K.dai=Kalanchoe daigremontiana; K.tubi=Kalanchoe tubiflora; L.esc=Lycopersicon esculentum; N=189bp enhancer fragment; N.glauca=Nicotiana glauca; SRB=synthetic right border.
Symbols: -, avirulent; +/-, weakly virulent; + and ++, partially virulent; +++, fully virulent.

virulence (LBA5235 and LBA5236 ; Table 1). To test whether overdrive can exert any stimulatory effect on transfer mediated by an inverted border repeat, we inserted a fragment containing overdrive both left as well as right of the right border repeats in LBA5251 and LBA5252.

This resulted in four strains, viz. LBA5265 and LBA5269 with a border repeat in the native orientation, and LBA5266 and LBA5270 with an inverted right border repeat (Table 1). As expected strains containing a Ti-plasmid with a synthetic right border repeat in the wild-type orientation together with the enhancer sequence (LBA5265 and LBA5269) induced wild-type tumour responses on all test plants three weeks after inoculation (Table 1). Strains LBA5266 and LBA5270 (inverted border repeat) turned out to be clearly more virulent than LBA5251, but were attenuated as compared to wild-type strains (Table I). These results show that the enhancer sequence can stimulate T-region transfer mediated by an inverted border repeat.

Production of single stranded T-molecules dependent on the orientation of the border repeat

To examine the mechanism underlying T-region transfer mediated by an inverted border repeat we looked for the production of T-strands in relevant strains after induction with acetosyringone. Southern blots were made of non-denatured gels containing undigested total DNAs isolated from these

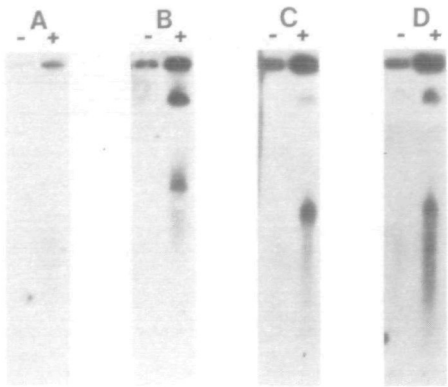

Fig. 1. Analysis of total DNAs prepared from acetosyringone induced (+) and non-induced (-) strain LBA5265-II. Similar results were obtained for strains LBA5266-II, LBA5251-II and LBA5252-II. The blots were probed for the top strand of the native T-region (A), the bottom strand of the native T-region (B), the top strand of the newly created T-region (C) and the bottom strand of the newly created T-region (D). Note that lanes A and B originate from a different gel than lanes C and D. All lanes were loaded with the same amount of DNA.
Abbreviations: Ti=Ti-plasmid; ss=single stranded T-molecules.

strains. Two bands were detected on these blots after hybridization with a T-region specific probe in the lanes containing DNA isolated from acetosyringone induced bacteria (Figure 1). Our results showed that only derivatives of LBA4417 with a border repeat inserted in their pAL4417 plasmid produced such bands. It is important to note that the occurrence of these bands turned out to be independent of the orientation of the right border repeat towards the onc-genes. Treatment of the DNA with S1-nuclease before loading the gel led to the disappearance of the bands, but RNase treatment had no effect as expected (data not shown). From these observations and the fact that the blots were made under non-denatured conditions, it can be concluded that the detected bands indeed represent DNA-molecules that are partly or entirely single-stranded. The upper bands hybridized also with DNA-fragments from the virulence region and replication region of the Ti-plasmid (data not shown), but the lower bands hybridized exclusively with T-region probes. It can thus be concluded that the lower bands represent single stranded T-molecules (T-strands) comparable to those described by Stachel et al. (15). The upper bands, which are not always present in DNA preparations from induced agrobacteria, apparently consist of Ti plasmids

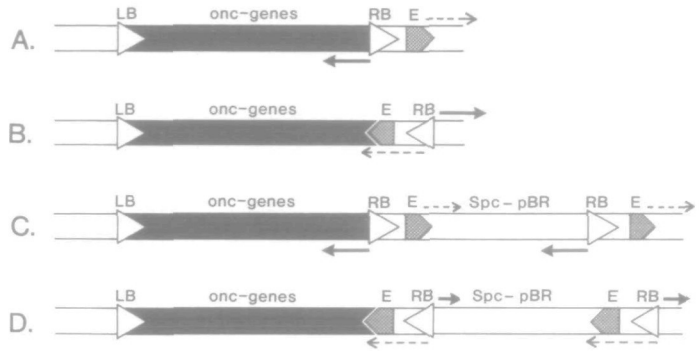

Fig. 2. Schematic drawing of the T-regions of strains LBA5265 (A), LBA 5266 (B), LBA5265-II (C) and LBA5266-II (D). The black bar indicates the native T-region with the onc-genes; the open bar indicates the newly formed T-region with the spectinomycin (Spc) resistance gene and the pBR-sequence originating from the shuttle vector pRAL5200. The arrows indicate the direction in which DNA synthesis occurs after border repeat nicking.
Abbreviations: LB=left border repeat; RB=right border repeat; E=enhancer sequence; Spc-pBR322=shuttle vector.

that have become single stranded partially and might represent molecules participating in the process of T-strand production.

By using strand specific DNA and RNA probes of the T-region we examined the nature of the detected single stranded T-molecules. Interestingly in all cases, for plasmids with a border repeat in the native orientation as well as for plasmids with a border repeat in the inverted orientation, T-strands could only be detected with probes representing the top strand of the T-region (Figure 1A,1B). Consequently, the single stranded T-molecules represent the bottom strand of the T-region. This result implicates that irrespective of the orientation of the right border repeat similar T-strands are produced in Agrobacterium.

Bidirectional transfer mediated by border repeats

With the constructs described above it was not well possible to analyse whether T-region transfer started on an inserted border repeat is bidirectional or only pointed in the direction of the onc-genes. However, strains that contain more than one insert of the (loaded) shuttle vector in tandem in pAL4417 contain extra, artificial T-regions to the right side of the native oncogenic T-region. These extra T-regions comprise the pBR322 sequences from the shuttle vector but no onc-genes between their border repeats (Figure 2). When a shuttle vector with an inverted border repeat is

introduced in multiple copies into pAL4417, extra, artificial T-regions are created that are situated in an inverted orientation as compared to the wild-type T-region. Similarly, shuttle vectors which introduce a border in direct repeat, create extra T-regions that are situated in the same orientation as the native T-region (Figure 2). Representatives of these two situations are the strains LBA5251-II, LBA5252-II, LBA5265-II and LBA5266-II, which are comparable to strains LBA5251, LBA5252, LBA5265 and LBA5266 but which contain two tandem inserts of the shuttle vector in their Ti-plasmid instead of one insert. These strains were used to assay whether transfer starting at a border repeat is unidirectional or bidirectional. Because of the extra border repeats present in these strains it becomes possible to detect the production of single stranded molecules outside the native T-region. Blots containing DNA isolated from acetosyringone induced or non-induced bacteria from these strains were hybridized with DNA and RNA probes specific for the top and bottom strands of the native T-region as well as the newly created T-regions (Figure 2). As shown in figure 1 both the top and bottom strand of the newly created T-regions are present in the induced bacteria as single stranded molecules (lanes C and D), whereas only the bottom strand of the native T-region is produced as a single stranded molecule (lanes A and B). These results show that in principle bidirectional transfer can indeed occur from a border repeat. However, since no top strands of the native T-region could be detected in our experiments (Figure 1; lane A), we have to conclude that from the native left border repeat bidirectional transfer does not occur at a measurable frequency.

DISCUSSION

A synthetic 24bp border repeat in combination with the enhancer sequence is sufficient to mediate T-region transfer to the plant cell with wild-type efficiency. The efficiency of DNA-transfer is not significantly influenced by the position and orientation of the enhancer sequence relative to the border repeat (23), but the orientation of the border repeat relative to the T-region is important (21). Although inverted border repeats are clearly less efficient in mediating DNA transfer to the plant cell than borders in the native orientation, transfer mediated by these inverted border repeats is still significant if an enhancer sequence is present next to the inverted border repeat.

Our results allow speculation on the mechanism underlying the transfer process mediated by inverted (and wild-type) border repeats. Three possible

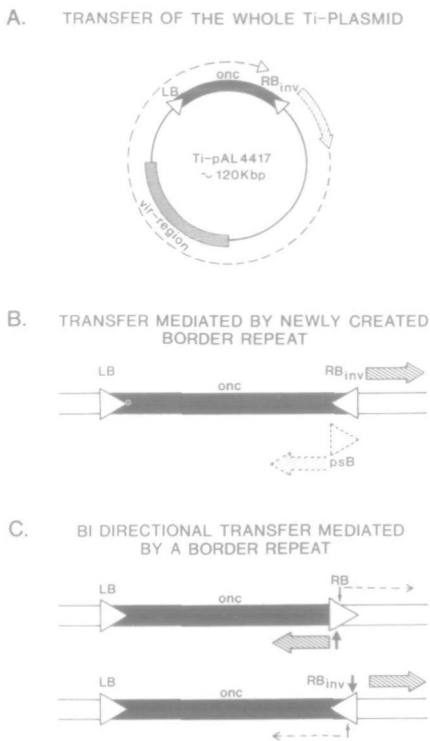

Fig. 3. Schematic drawing of three models for DNA transfer starting on an inverted right border repeat. The arrows indicate the direction in which DNA synthesis proceeds.
Abbreviations: LB=left border repeat; RB=right border repeat; RBinv=right border repeat inverted; psB=pseudo border repeat; onc=oncogenicity genes; vir=virulence region.

processes might explain the relatively efficient transfer mediated by inverted border repeats (Figure 3).

First the border repeat might be able to mediate transfer of the complete pAL4417 plasmid (120Kbp) starting at the inverted border repeat and mediating transfer to the right, away from the onc-genes (Figure 3a). One has to assume that the left border repeat of pAL4417 is not recognized in this case as an end point of transfer and that, consequently, the onc-genes are transferred to the plant cell. This scenario is highly unlikely, however, because Joos et al. (18) have shown that when the onc-genes are inserted elsewhere in the Ti plasmid at a distance from the T-region, transfer of the onc-genes to the plant cell occurs only at a much decreased level.

A second possibility is that the insertion of fragments with a 24 base pair repeat in pAL4417 creates a pseudo border repeat on the place of the insertion that is again in direct repeat with the left border repeat of pAL4417 and therefore can mediate the transfer of the onc-genes to the plant cell with relatively high frequencies (Figure 3b). To circumvent problems with possible interfering sequences such as pseudo border repeats we used synthetic border repeats in these studies. Although there is some inner symmetry inside the border repeats, in both cases the homology of the created pseudo border repeat with the border repeat itself is very poor (less than 50% homology). Therefore, insertion of a synthetic border repeat in the inverted orientation, cannot acccount for the frequencies of T-region transfer as detected here for inverted border repeats.

Based on our results we propose a third model in which bidirectional transfer occurs, starting at a border repeat. This is in agreement with the fact that during T-DNA processing not only nicking occurs in the bottom strand of the border repeat, but also albeit less efficiently in the top strand of the border repeat (12). If this occurs in inverted border repeats as well, it can easily be seen that this will lead to nicks in the bottom strand that can function as a start site for the production of T-strands representing the bottom strand (Figure 3c). In agreement with this we could indeed show the production of T-molecules representing both top and bottom strand in our assays. This indicates that bidirectional transfer can be started on border repeats, which is in agreement with the above presented model. Our data also show that tumorigenicity is increased in strains with an inverted right border by the presence of the T-DNA transfer enhancer. The function of the enhancer is to stimulate T-strand formation (23) and together this suggests that the formation of T-strands is favoured in both directions by the presence of the enhancer. Bidirectional transfer does not seem to occur from the native left border repeat. This can be deduced from the fact that no top strand intermediates representing the T-region were found in acetosyringone induced bacteria. This may be due to the absence of an enhancer next to this border repeat, or to the presence of a sequence inhibiting T-strand formation near this repeat.

ACKNOWLEDGEMENTS

We thank Drs.L.Melchers, K.Rodenburg and R. van Veen for critically reading the manuscript. This work was supported by the Netherlands Foundation of Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Scientific Research (ZWO).

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