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Recovery of Agrobacterium tumefaciens T-DNA Molecules from Whole Plants Early after Transfer

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Summary

A system for the analysis of independent T-DNA transfer events from Agrobacterium to plants is described. The complete T-DNA except for the 25 bp border sequences was replaced by one genome of a plant virus so that upon transfer to the plant, a viable replicon is produced by circularization. Rescue of virus from such infected plants allowed analysis of DNA sequences at or close to the ends of T-DNA molecules. A rather conserved right border remnant of three nucleotides was found, whereas the sequences remaining at the left end were more variable. A point deletion in the left 25 bp sequence results in even less precise processing at the left end. In addition, many rescued T-DNA molecules carry small direct repeats between the joined T-DNA ends; linear T-DNA molecules are therefore transported to the plant.

Introduction

Agrobacterium tumefaciens, a soil-born plant pathogen, can infect most dicotyledonous plants at wound sites, causing a tumorous outgrowth of transformed plant cells called crown gall. The transforming genes of Agrobacterium reside on a stretch of DNA called transfer- or T-DNA, which is part of a 200 kb tumor-inducing (Ti) plasmid. The T-DNA carries, among others, tumor genes for the production of plant growth factors, which are under the control of eukaryotic promoter signals acting only in plants. On the Ti-plasmid, the T-DNA is defined and flanked by two 25 bp imperfect direct repeats called border sequences (for reviews, see Gheysen et al., 1985; Koukolíková-Nicola et al., 1987; Melchers and Hooykaas, 1987).

The virulence (*vir*-)region, a stretch of about 23 kb, is the other region on the Ti-plasmid required for tumor formation. It encodes genes that fall into seven complementation groups, designated *vir*A through F. Their expression is tightly regulated by *vir*A and *vir*G in response to substances such as acetosyringone produced by wounded plant cells (see Stachel and Zambryski, 1986, for review). Two genes of the *vir*D locus were found to be responsible for processing the T-DNA in the bacterium (Alt-Moerbe et al., 1986; Yanofsky et al., 1986; Stachel et al., 1987; Yamamoto et al., 1987; Veluthambi et al., 1987). Hoekema et al. (1983) showed that the *vir*-region can act in *trans* on the T-DNA, allowing the separation of these two entities onto two independent replicons, producing the so-called "binary vector system."

Induction of the virulence genes leads to processing of the T-DNA at the border sequences. Thereby specific nicks in the lower strand (Stachel et al., 1986; Yanofsky et al., 1986; Albright et al., 1987; Stachel et al., 1987; Wang et al., 1987) and single-stranded T-DNA molecules of lower strand polarity, the so-called T-strands, are produced (Stachel et al., 1986; Albright et al., 1987; Veluthambi et al., 1987; Stachel et al., 1987). Double-stranded cuts at the borders have also been found (Albright et al., 1987; Veluthambi et al., 1987; Dürrenberger et al., unpublished data), whereas a very small fraction forms covalently closed T-DNA circles in vivo (Machida et al., 1986; Timmerman et al., 1988). In such circles the T-DNA ends are precisely joined within the right and left 25 bp sequence, resulting in a hybrid sequence, as has been found for induced T-DNA molecules rescued by lambda in vitro packaging (Koukolíková-Nicola et al., 1985). The involvement of all the molecules described above in T-DNA transfer is not clear.

Following transfer to plants by a largely unexplored mechanism, the T-DNA is integrated into the plant genomic DNA. The T-DNA–plant DNA junctions have been mapped within or in the vicinity of the border sequences (Yadav et al., 1982; Zambryski et al., 1982; Simpson et al., 1982; Holsters et al., 1983; Kwok et al., 1985). However, very few integrated copies have been studied in detail, and, except in one case (Gheysen et al., 1987), only one end of each integrated T-DNA was analyzed.

By replacing the T-DNA by a plant replicon, we set up a system that allows observation of many independent transfer events. Figure 1 schematically depicts the experimental setup. As plant replicon we use cauliflower mosaic virus (CaMV), which contains an 8 kb long double-stranded circular DNA genome (for review, see Gronenborn et al., 1987). It is linearized in its nonessential gene II, placed on a binary plasmid between synthetic 25 bp T-DNA border sequences, and introduced into Agrobacterium containing a helper Ti-plasmid. Upon agroinfection (Grimsley et al., 1986) of Brassica campestris variety rapa, a host plant for both Agrobacterium and CaMV, with these engineered Agrobacteria, T-DNA intermediate molecules consisting of one viral genome flanked by processed border sequences are produced and transferred. Instead of (or in addition to) integration into the plant nuclear genome, joining of viral DNA ends occurs, producing a circular infectious viral molecule. Replication of the reconstituted viral genome leads to the appearance of symptoms. Thus, individual transfer events are amplified and can easily be recovered. Analysis of viral DNA from systemically infected leaves, particularly of the T-DNA end-junction in its gene II region, allows deductions on the structure of the transferred T-DNA molecules.

Results

Constructions

CaMV mutants were cloned in between synthetic nopaline

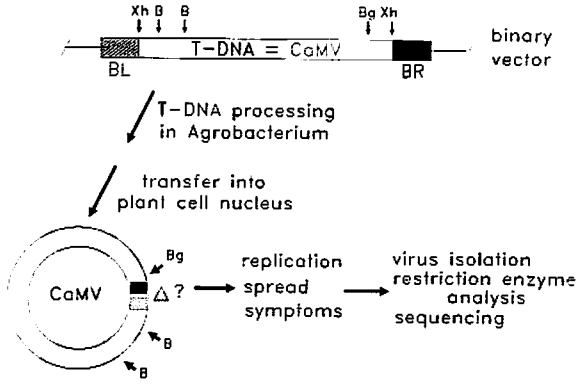

Figure 1. Schematic Representation of the Experimental Design, Chain of Events, and Analysis Procedure (Not to Scale)

The T-DNA is replaced by one genome of CaMV = *Cauliflower Mosaic Virus*. BL = left border; BR = right border. Relevant restriction sites are: B = BamHI; Bg = BglII; Xh=XhoI. Upon arrival in the plant cell nucleus, the molecule is circularized at the end-joining region (= Δ) and reconstitutes an infectious viral genome.

C58 Ti-plasmid-derived 25 bp border sequences residing on a broad host-range plasmid pRK252. Cloning was done in the CaMV gene II region, which has a unique XhoI site and is dispensable. Since we used a replicon with a biological lifecycle of its own, we had to consider several factors. It was not known if the CaMV "relay-race model" for translation, which requires a start codon soon after a stop codon (Dixon et al., 1984), would impose limitations on the kind of DNA sequences we might be able to rescue from plants. Therefore we used CaMV deletion mutants Ca312, which has an ATG creating a small ORF II of 27 bp, and Ca355, which lacks a start codon thereby leaving a small intergenic region of 12 bp in place of gene II. In order to reduce possible limitations of a small gene II region, we have also used CaMV mutant Ca3, which has an almost wild-type ORF II of 480 bp and therefore more nonessential sequences on both sides of the XhoI cloning site ("playground"). Figure 2 shows the different binary pEAP (plasmid–*E. coli*–Agrobacterium–plant) constructs used. Ca355 and Ca3 were cloned in between synthetic nopaline wild-type border sequences, yielding pEAP134 and pEAP145, respectively. Ca355 and Ca312 were also cloned in two orientations in between synthetic border sequences, of which the left border repeat had a deletion of one base pair in the core sequence. This gave the border mutant constructs pEAP104, 105, and 112, 113 respectively.

Reconstruction Experiments

To use the viral replicon to rescue T-DNA border remnants from plant cells, we first needed to test whether borders or border fragments would be tolerated and maintained in the CaMV gene II region, whether in vivo exonucleolytic degradation of the transferred DNA molecules would modify, and thereby obscure, the T-DNA specific ends, and whether the binary constructs used would also be infectious as DNA and thereby produce background infectivity not caused by agrobacterial transfer. We therefore conducted three sets of reconstruction experiments designed to test for possible interference in our assay system that might be caused by factors not related to T-DNA transfer.

CaMV Is a Faithful Vector for T-DNA Border-Derived Sequences

Should circular T-DNA molecules be the infectious T-DNA intermediates, perfect hybrid T-DNA borders would be expected to enter the plant (Koukolíková-Nicola et al., 1985; Machida et al., 1986). Such hybrid borders served as models to test the faithfulness of the CaMV cloning system, which otherwise has been documented for the cloning and expression of the genes for methotrexate resistance (Brisson et al., 1984) and interferon (de Zoeten et al., submitted). The plasmids pCa355 and pCa312 contain the CaMV mutant genomes Ca355 and Ca312 (see above) cloned in the SalI site of the essential ORF V (see Figure 2). Plasmids pCa355THB1 and pCa355THB2, and

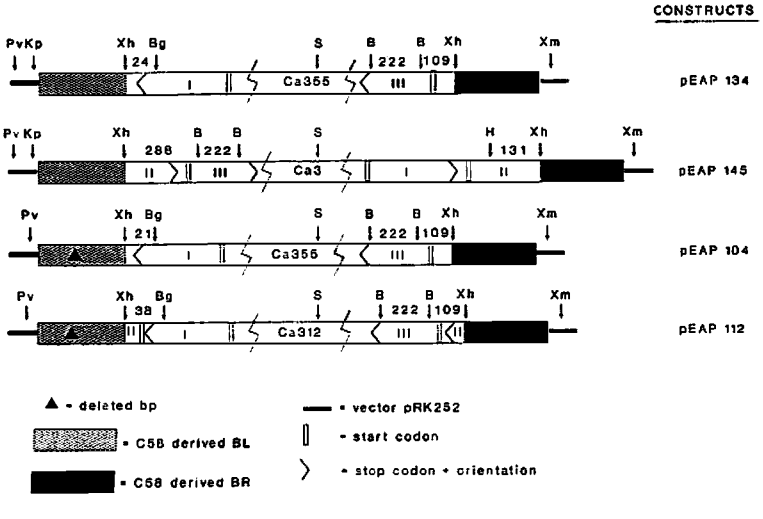

Figure 2. Constructs Used for Agroinfection

Components are not drawn to scale. pEAP104 with Ca355 cloned in inverted orientation is pEAP105; pEAP112 with Ca312 in inverted orientation is pEAP113. Only relevant restriction sites are marked. Pv=PvuI; Bg=BglII; B=BamHI; Xm=XmaI; Kp=KpnI; H=HindIII; Xh=XhoI; S=SalI. Roman numerals refer to the CaMV ORFs; for clarity the ORFs IV through VII are omitted. Numbers above the constructs give the length in bp between the indicated restriction sites.

Table 1. Infectivity of Plasmid DNA

DNA Sample	"Playground"	No. Systemically Infected Plants / No. Inoculated Plants
SalI-restricted:		
pCa355 (parent)	12	3/4
pCa355THB1	51	4/4
pCa355THB2	51	5/8
pCa312THB1	68	2/8
pCa312THB2	68	4/4
XhoI-restricted:		
pEAP104, pEAP105, pEAP134 (= Ca355)	12	7/12
pEAP112, pEAP113 (= Ca312)	29	4/8
pEAP145 (= Ca3)	482	4/4
Unrestricted:		
pEAP104		0/8
pEAP134		0/8
pEAP145		2/8

Inoculation series are separated by lines. "SalI-" and "XhoI-restricted": plasmid DNA was restricted with the respective enzymes prior to inoculation. "Playground": the amount of nonessential base pairs in the CaMV ORF II region around the XhoI cloning site. See Figure 2 for a description of constructs.

pCa312THB1 and pCa312THB2 contain a synthetic 25 bp hybrid border sequence in the unique XhoI site in the gene II region of pCa355 and pCa312, respectively, in both orientations. One genome of CaMV cloned in an E. coli vector is not normally infectious when inoculated on leaves of host plants unless the DNA is first excised from the vector at the cloning site (Lebeurier et al., 1980). Cleavage of the respective hybrid border–containing pCa mutant genomes with SalI yielded the linear DNA used for inoculation. Reconstitution of the SalI site in vivo generated viable, circular CaMV genomes. As shown in Table 1, all four constructs were equally infectious and comparable in infectivity to pCa355, the CaMV parent strain used. Analysis of the ORF II region of progeny virus reveals a precise conservation of insert sequences (Figure 3B). The inoculum DNA (lanes 1 and 15) and viral DNA isolated from individual infected plants (lanes 2, 3, and 14) were restricted with BamHI, end-labeled, further restricted with BglII (see Figure 1), and analyzed by electrophoresis on an 8% polyacrylamide gel. In addition to the constant 222 bp BamHI–BamHI fragment, a 164 bp band is observed for all Ca355THB-derived (lanes 1, 2, and 3), and a 181 bp band is observed for Ca312THB-derived (lanes 14 and 15) input and progeny virus. These latter fragments encompass the cloned hybrid border sequence. Its identity was confirmed by sequencing of three samples of progeny virus. Thus, the hybrid border, as an example of border-derived sequences that one might expect to recover from plants, was faithfully maintained in its vector CaMV.

Exonucleolytic Degradation of Infecting Viral DNA Is Not Observed

CaMV DNA linearized in the middle of its dispensable gene II region by restriction with XhoI served as an indicator for possible exonucleolytic degradation within the plant upon inoculation, as 12, 29, and 482 bp of Ca355, Ca312, and Ca3, respectively, can be degraded without loss of infectivity. Inoculation this time was with DNA from the respective pEAP-constructs restricted with XhoI, which separates the vector from the viral genome (see Figure 2). Although there is no selection on exact ligation, DNA isolates from 15 independent successful inoculations (Table 1) showed reconstitution of the XhoI site. In addition, in three cases tested, the DNA sequence surrounding the XhoI site was found to be identical to the inoculum sequence (data not shown). However, we cannot rule out the presence of a highly processive nuclease that degrades a proportion of the incoming molecules extensively, producing inviable virus. Since the progeny virus within one plant may have arisen from more than a single reconstitution event, the actual number of independent ligations was probably higher. The latter interpretation might be supported by the observation that within one plant tested a fraction of the virus DNA was found to contain a replacement of 13 viral bp including the XhoI site by 22 bp of unknown origin.

The Binary Constructs Are Several Orders of Magnitude Less Infectious when Applied as DNA than when Transferred by Agroinfection

In the binary constructs employed in this study, the viral genome is flanked by 25 bp direct although imperfect repeats, which may provide sufficient homology for recombination to generate infectious molecules independently of T-DNA processing at the borders. If DNA molecules longer than one complete T-DNA were to be transferred upon agroinfection, they could rearrange by homologous or illegitimate recombination to produce infectious virus. However, out of 24 plants inoculated with naked DNA only 2 yielded symptoms; in both cases the plants had been inoculated with DNA construct pEAP145 (Table 1). In one plant the virus had recombined out via the

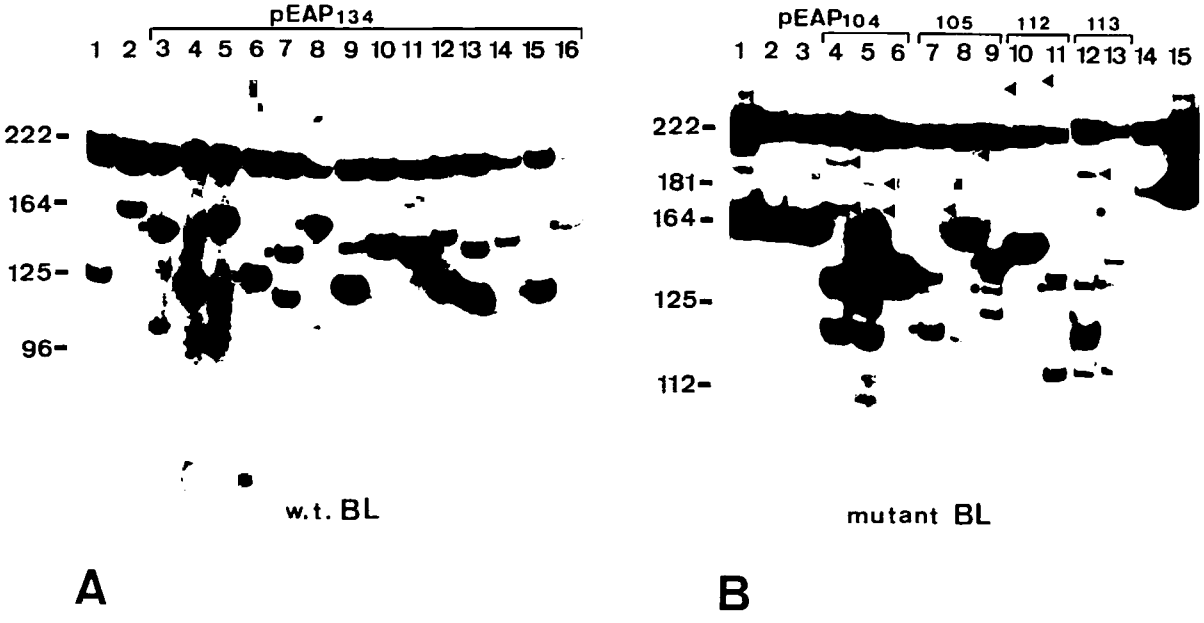

Figure 3. Analysis of T-DNAs Isolated from Different Plants

Radioactive end-labeled BamHI–BglII and BamHI–BamHI fragments were separated on an 8% polyacrylamide gel (see Experimental Procedures). Fragment sizes are in bp; note the 222 bp virus internal BamHI–BamHI fragment.

(A) Lane 1: plasmid pCa355; lane 2: plasmid pCa355THB2. Other lanes show viral isolates from different systemically infected plants (one lane corresponds to one plant), all inoculated with A. tumefaciens GV3105(pEAP134). Lanes 3–8: crown inoculated plants. Next lanes: leaf inoculation with an inoculum of 10^8 (lanes 9, 10, and 11), 10^7 (lanes 12, 13, and 14), and 10^6 (lanes 15 and 16) bacteria per plant.

(B) CaMV-derived from the BL point deletion mutant constructs and from hybrid border reconstruction experiments. Lane 1: plasmid pCa355THB2; lanes 2 and 3: viral isolates from plants inoculated with plasmids pCa355THB1 and pCa355THB2 respectively, both SalI-digested before inoculation; lanes 4–13: viral isolates from systemically infected plants (one lane corresponds to one plant), inoculated on the leaves with A. tumefaciens strain LBA4404 containing binary pEAP constructs as indicated. Lane 14: viral isolate from a plant inoculated with SalI-digested plasmid pCa312THB2; lane 15: plasmid pCa312THB1. Triangles point to fragments sensitive to PvuI (compare Figures 2 and 4). A dot on the left side of a band indicates a fragment that has been sequenced (see Figure 5).

two border repeats, yielding a perfect hybrid border, and in the other plant two CaMV ORF II region fragments were seen in the gel assay, indicating the presence of two new viral mutants (data not shown). In the latter case both types of virus had been produced by a recombinational event between one of the border repeats and an internal part of ORF II, creating deletions of about 235 and 325 bp, respectively.

It should be kept in mind that the DNA inocula consisted of 10 μg per plant, whereas 10^8 agrobacteria per plant, carrying the same constructs, were fully infectious (see below). This is a 10^4- to 10^5-fold difference when expressed as infectious DNA equivalents. Thus, we conclude that viral DNA molecules replicating in the plant using our agroinfection system are the result of agrobacterial processing at the T-DNA border sequences and subsequent transfer of T-DNA.

Analysis of T-DNA Transfer

Efficiency

Constructs described in Figure 2 were introduced into various Agrobacterium strains and inoculated on turnip plants. Table 2 shows examples of several sets of comparisons, divided by lines. Because of differences between plant batches, comparisons are possible only within a set. Constructions used for the first set differed in the extent of ORF II. pEAP145 contains a complete gene II region and in principle has more space ("playground") for recombination, degradation, or other rearrangements adjacent to the T-DNA borders. pEAP134 is equally agroinfectious in this test even though it contains only 12 bases of gene II region. This might indicate that bona fide T-DNA transfer events are observed. Neither the orientation of the CaMV genome (lines 7 versus 8 and 9 versus 10) nor the presence of a minigene II (defined by the ATG codon, constructs pEAP112 and 113) influences the agroinfection efficiency, in comparison to constructs containing an intergenic region (pEAP104 and 105). We conclude that the events observed in our assay will reflect T-DNA rather than CaMV biology.

Nopaline strains are more efficient in transfer than octopine strains (lines 5 and 6). This may be because virulence proteins of an octopine strain are produced at lower levels than those of a nopaline strain (Engstrom et al., 1987), or because octopine strains have a *more stringent* requirement for an "overdrive" sequence, a T-region transfer enhancer found in octopine strains to the right of the right border repeat (Peralta et al., 1986). Our constructs do not have an obvious "overdrive" sequence (see Discus-

Table 2. Efficiency of Agroinfectivity (Representative Examples of Many Experiments)

No.	A. tumefaciens strain	Binary pEAP#	No. Systemically Infected Plants / No. Inoculated Plants
1	nop	134	4/4
2	nop	145	4/4
3	nop	134	4/4
4	nop	104	0/4
5	oct	134	0/4
6	nop	134	3/4
7	oct	104	3/4
8	oct	105	3/4
9	oct	112	3/3
10	oct	113	2/4

Series between lines cannot be compared with other series because of changes in sensitivity between different plant batches. Zero infectivity means not showing symptoms 35 days post inoculation. nop = nopaline host GV3105; oct = octopine host LBA4404. Approximately 10^8 bacteria were used per leaf inoculum.

sion). At higher inocula, however, also octopine strains yielded symptoms (data not shown).

Finally, the influence of a border mutation was tested. The two constructs pEAP134 and pEAP104 differ by a single base pair, which is missing from the core of the left border in pEAP104. The efficiency of agroinfection of the mutant was strongly reduced in comparison to the wild type (Table 2, rows 3 and 4). This was surprising because an effect of the left border on efficiency of T-DNA transfer has so far not been reported. Symptoms appeared only when higher concentrations of bacterial inocula were used.

Taken together, the reconstruction experiments indicate that the CaMV replicon allows faithful and stable cloning of T-DNA transfer-specific sequences. In the next two sections their analysis in terms of size and actual base composition is presented.

Size Variations of T-DNAs Isolated from Plants

Viral DNAs were isolated from systemically infected leaves and this original, uncloned DNA was subjected to restriction enzyme analysis in a way that mainly shows fragments containing the junction region on gels (see previous section). Figure 3A reveals small variations in the junction fragment sizes. These small size differences are found within and between individual plants. The very existence of this size variation is an argument in favor of recovery of individual T-DNA transfer events. No great differences in size or number of junction fragments could be detected between plants that had been inoculated on the crown (lanes 3–8) or on the leaves (lanes 9–16). The inocula on the crown (10^5–10^6 bacteria/plant) are much smaller than the ones used on leaves (10^8 bacteria/plant), and this may explain the smaller number of different junction fragments in any one crown-inoculated plant. The trend with decreasing inoculum size in the leaf-inoculated series is in the same direction.

Most of the fragments have sizes similar to (164 bp) and smaller than the ones containing a perfect hybrid border (plasmid control in lane 2, Figure 3A). However, fragments smaller than 113 bp could not have been detected because the corresponding deletions would have reached into essential regions of the virus. To overcome this potential selective disadvantage, inoculations were repeated using the construct pEAP145, which offers a "playground" of 296 and 186 bp adjacent to the right and left border, respectively, which could be deleted without loss of viability (Figure 2). However, the general distribution of sizes found in junction fragments was similar with some cases of about 100 bp deleted (data not shown).

Strains with a deletion in the left border showed a large variation in fragment sizes (Figure 3B, lanes 4–13). Junction fragments resulting from constructs pEAP104 and 105 should be compared with the corresponding fragment of 164 bp of Ca355THB (lanes 1–3); those from constructs pEAP112 and 113 with the 181 bp fragment of Ca312THB (lanes 14, 15). Again, there are different patterns in each plant, but there are more individual bands per plant than are seen in Figure 3A. In addition, fragments larger than the perfect hybrid border are seen in most plants (labeled by small filled arrows). This points to a less precise processing step in the bacterium than in the wild-type border situation.

In the mutant case, incorrect border processing might have led to inclusion of sequences from the binary vector adjacent to the T-DNA left border. To test this hypothesis, the junction-specific BamHI–BglII fragments were subjected to additional restrictions with PvuI or XmaI. These enzymes have unique recognition sequences 2 bp left of the left border and immediately right of the right border, respectively (Figure 2), and susceptibility of a fragment to one of these enzymes is therefore indicative of transfer of "outside" T-DNA sequences. Figure 4 shows such an analysis for three different virus isolates generated by the border mutant constructs. All fragments of sizes larger than the 25 bp hybrid border standard of 164 bp (lane 1) disappear upon PvuI cleavage (filled triangles), but resist the action of XmaI. This indicates that sequences flanking only the left mutated border have entered the plants. In contrast, restriction of pEAP134 and pEAP145 wild-type-derived virus isolates did not reveal any bands sensitive to KpnI, which has a unique recognition sequence just to

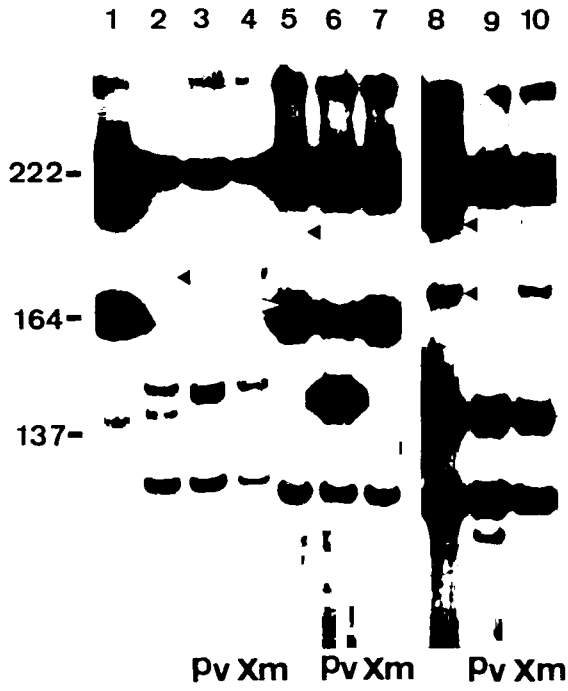

Figure 4. Test for Sequences outside of the Borders

PvuI and XmaI sensitivity is an indication for the presence of sequences beyond the left and right border, respectively (see Figure 2). Viral DNA was treated as described in the legend of Figure 3, except for an additional restriction with PvuI (lanes 3, 6, 9), or XmaI (lanes 4, 7, 10). Lane 1: plasmid pCa355THB1; lane 2 compares to Figure 3B, lane 7; lane 5 compares to Figure 3B, lane 8; and lane 8 compares to Figure 3B, lane 4. Triangles point to fragments sensitive to PvuI. Fragment sizes in bp.

the left of the wild-type left border (see Figure 2; data not shown).

Since the viral genomes for the sequence analysis were produced from the deletion mutant constructs in combination with the octopine strain LBA4404 and from the wild-type border constructs in combination with the nopaline strain GV3105, the reciprocal combinations GV3105(pEAP-104) and LBA4404(pEAP134) were also tested. The bacterial background was shown not to influence the results as far as the restriction enzyme pattern in the gel assay was concerned. The effect of the border mutation thus seems to be two-fold: strains carrying it show a reduced level of T-DNA transfer (Table 2), and those molecules that are found in the plant indicate less precise processing at the left border.

Sequence Analysis of T-DNA Junctions

The nature of the junction region was examined by cloning and sequencing 47 randomly chosen virus isolates from 24 different plants agroinfected with all six described constructs. The sequences of 29 T-DNA end-joining regions are compiled in Figure 5B in a linear form, opened at the position of the junction, and aligned with the T-DNA sequence as present in the bacterium (5A). The right and left border sequences in 5A are linked to vector sequences, whereas the right and left border remnants from the T-DNAs rescued from plants are linked to each other. Note the change in scale: the center (shaded area) represents the 7.6–8 kb of CaMV-T-DNA, whereas border sequences, linkers between virus DNA and borders, and some sequences left of the left border, are shown by the base, either as bases (border sequences) or as dots (other nucleotides). The open triangles indicate the positions of the nicks found in the lower strand following virulence induction (Albright et al., 1987; Wang et al., 1987; Dürrenberger et al., unpublished data). The lengths of the vertical bars in Figure 5A represent the frequency with which a certain base pair was found to be an endpoint of a T-DNA; bars pointing down relate to ends of wild-type border constructs; bars pointing up to the mutant series. Nucleotides in parentheses (Figure 5B, see below) are redundant and cannot be assigned to either the left or the right border end. They have been counted at both T-DNA ends, with half frequency each. However, only one such sequence should be counted per DNA isolate. The lowercase letters refer to the respective fragments on the gels of Figures 3A and 3B, which have been sequenced (indicated with a dot on their left side, counting in the gels from left to right, and from large to small; no gel shown for pEAP145-derived fragments). The dotted line divides sequences resulting from the wild-type constructs (upper part) and mutant constructs (lower part). T-DNAs found within one plant are grouped by inside brackets.

Out of the other 18 sequenced isolates, 8 can be explained as arising from recombination at the XhoI site (Figure 2), and 10 may have arisen by recombination from larger viral molecules within one plant, although no obvious homologies that could have served as target for recombination were found. In these cases the largest sequenced molecule is given in Figure 5B. Eight of this group of ten are included as open bars in Figure 5A; the remaining two are not shown because their endpoints lie outside of the stretch of DNA included in the figure. This does not exclude the possibility that some of the molecules shown in Figure 5B are derivatives of larger precursors. For instance, the molecules with 1–4 redundant bases could also be recombination derivatives.

Several points should be noted. First, there is a striking absence of nucleotides to the right of base 3 in the right border. This contrasts with the variation found at the left end of the T-DNA. These results are in agreement with fine restriction enzyme mapping of T-DNA–plant DNA junctions (Jorgensen et al., 1987). Second, sequences with a perfect hybrid border were not found. They would have been expected if circular molecules had entered the plant. Third, at the junctions of roughly every third end, 1–14 bases of unknown origin are found (see Discussion), marked in < > parentheses in Figure 5B. Fourth, constructs with a left border point deletion give rise to T-DNA molecules with an even greater variability in the left end, frequently including a stretch of vector sequence. This is consistent with the restriction enzyme analysis of uncloned border junctions as presented in Figure 4.

Discussion

The Approach

We have described a system that amplifies single T-DNA

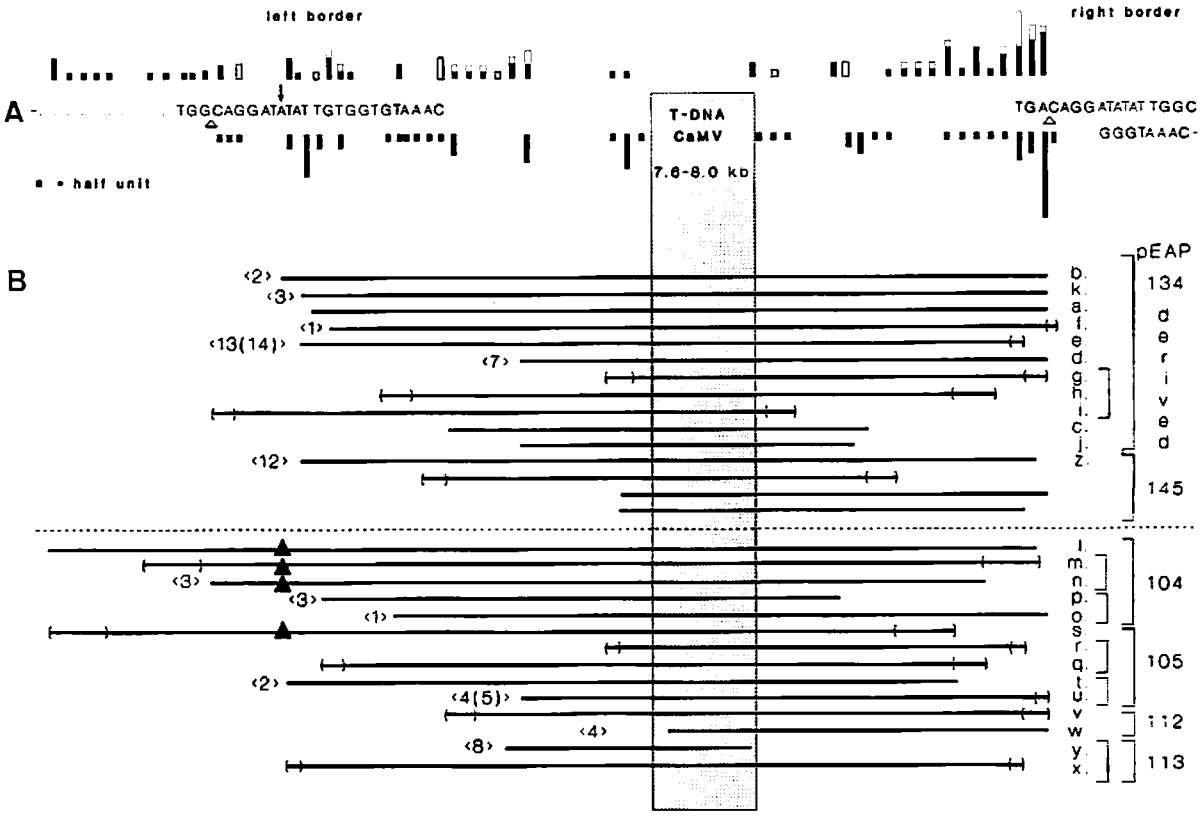

Figure 5. Compilation of Representative T-DNA Molecules Isolated from Plants

(A) represents the relevant sequence of the upper strand of all binary constructs used. The vertical filled arrow points to the A in the left border sequence, which is deleted in the border mutant. The open triangles point to the position of the nick introduced in the lower strand upon induction of the Agrobacterium *vir* region. For further explanation, see text.

transfer events in a plant and thereby allows recovery of Agrobacterium tumefaciens T-DNA molecules early after their transfer. In the experiments described here, efficient transfer occurs even in the absence of the "overdrive" sequence. However, the lower efficiency observed when using an octopine Ti-plasmid as a helper instead of a nopaline Ti-plasmid might be due to the absence of such a sequence in our binary constructs. The possible biological activity of a sequence with a fortuitous homology to overdrive (GAAACCCTTAGTATGTATTTGTATTTG), located about 820 bp upstream of ORF I of CaMV (map position 7564–7590 of CaMV-1841) was not tested.

Control inoculation with unrestricted binary vector DNA gave few or no infected plants, whereas agroinfection was efficient, independent of the orientation of the CaMV genome with respect to the borders, and independent of the presence or absence of an ORF II, either small or wild-type in size. These results, in combination with the demonstration of the absolute stability in the plant of a hybrid border sequence cloned in the CaMV ORF II region, suggest that the influence of the CaMV "replication biology" on the system is minimal.

Integration of the CaMV-T-DNA is probably not required to start the viral life cycle, because the viral genome would be disrupted. Genomic RNA, necessary for viral replication (CaMV reverse transcription review; Hohn et al., 1985), could form only from an integrated tandem dimer or multimer.

Characterization of the T-DNA Transferred to Plants

Two arguments suggest that the transferred intermediate is a linear molecule. First, out of 47 sequenced T-DNA molecules isolated from plants, none showed a perfect 25 bp hybrid border. Such hybrid border sequences would have been expected, if T-DNA molecules such as those rescued from induced agrobacteria were transfer intermediates (Koukolíková-Nicola et al., 1985; Machida et al., 1986; Timmerman et al., 1988). Second, the additional nucleotides we have found in the end-joining region with no exception appeared between bases remaining from the left and right border. If circular molecules were transferred to plants, their heterogeneity at the border joints would have to be explained as having arisen already in the bacterium, which has never been observed. Some heterogeneity has been reported from Agrobacterium-derived T-DNA circular molecules, but in these rare cases (illegitimate) recombination between sequences outside the T-DNA was involved (Timmerman et al., 1988). We favor the model discussed below, namely that the additional bases are byproducts of the circularization event in the

plant. Whether single- or double-stranded T-DNA molecules are transferred from Agrobacterium to plant cannot be differentiated by our experiments.

In induced agrobacterial cells, the lower strand is nicked at the third or fourth base of both borders (Albright et al., 1987; Wang et al., 1987; Dürrenberger et al., unpublished data), indicated with triangles in Figure 5A, which defines the ends of the T-DNA molecules. Comparison of the ends of the plant-derived T-DNA molecules with those of T-strands or double-stranded T-DNA molecules found in induced agrobacterial cells shows that they are similar but not identical. In our studies there are no bases past the third base of the right border passed on to the plant, and, in contrast to the left border, the position of the right end is better conserved. The few previously analyzed integrated T-DNA copies yielded similar results, although it should be kept in mind that information on both ends of single T-DNA molecules is missing, and comparisons of right and left ends are therefore not so conclusive (reviewed in Koukolíková-Nicola et al., 1987). Our results on the relatively well conserved right T-DNA ends suggest that they may be protected from degradation during transfer. Indeed, the *virD2* protein is found firmly attached to the 5′ end of the nicked lower T-DNA strand in Agrobacterium (Young and Nester, 1988; Dürrenberger et al., unpublished data). We suggest that this protein accompanies or even guides the T-DNA to the plant nucleus where it is cleaved off. The protection of the right end and polarity of T-DNA transfer from this end may be biologically meaningful because the genes close to the right end are the ones that mostly direct the synthesis and secretion of the opines, the nutrients for the agrobacterial cell.

A point deletion in the left border leads to a decreased transfer efficiency and increased variation in the position of the left end: whereas no base is found beyond the position of the lower strand nick in the wild-type left border, several of the sequenced isolates and many of the unsequenced ones (see Figure 4) show overriding of the mutant left border. In the mutant, a base in the conserved core sequence is deleted. The consequence could be a weaker binding of the *virD* endonuclease to the mutated recognition sequence, leading to a less efficient processing or no processing at all, thereby creating longer, aberrant T-DNA molecules. Indeed, use of the right border se-

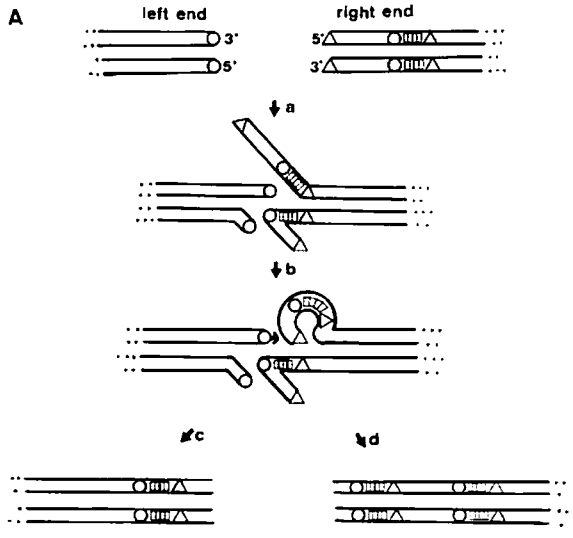

Figure 6. The Slip-Mispair-Repair Model (Roth et al., 1985) As a Possible Mechanism by Which the T-DNA Molecules Could Have Circularized

The open circles and triangles indicate short sequence homologies. See text for further explanation. In the left panel of (B), examples of the base composition of the junction region (ends plus additional base pairs) of four T-DNAs (refer to Figure 5B) are shown. Arrows indicate the direction a particular terminal base (dot) slipped to find a homology in the CaMV genome, shown in the right panel. Numbers adjacent to the dot indicate the distance between the two homologies. In the right column, the extent of the homology between bases found in the junction region and the CaMV sequences are shown. Underlining indicates the number of bases inserted in the end-joining reaction.

quence only would probably allow transfer of the whole binary construct to the plant cell, as was found for binary vectors carrying only one border sequence (Jen and Chilton, 1986a, 1986b). Such molecules would give rise to nonviable viral genomes. Only molecules that are broken or shortened due to nuclease attack or illegitimate recombination can replicate and produce viral symptoms. This might explain the increase in heterogeneity at the left and possibly the right border end. However, we cannot exclude that the slight increase in heterogeneity at the right border end could be due to the lack of an "overdrive" sequence, since the mutant constructs were used in combination with an octopine *vir* region.

Does Circularization Show Aspects of T-DNA Integration?

As stated above, the reconstruction experiments permit the conclusion that the CaMV-T-DNA molecules we analyzed represent T-DNA molecules with little or no influence of CaMV biology on the primary T-DNA structure. Peculiarities observed at the junction are therefore a consequence of the end-joining reaction. At least some of the molecular interactions that occur in circularization are likely to be the same as those involved in integrating T-DNA in plant genomic DNA. In the natural situation the T-DNA probably does not go through a circularization step. However, the high number of only transiently transformed plant clones reported by van Lijsebettens et al. (1986) may be explained by expression of T-DNA genes from a circular copy that later aborts.

In theory, a linear DNA molecule has two options for circularization in a cell: direct end-to-end ligation and recombination. Whereas the first is most likely responsible for the perfect joining of the ends generated by XhoI digestion of naked viral DNA in the reconstruction experiment, both mechanisms could be responsible for joining T-DNA ends in the plant. Could the bases we found inserted between the joined remnant border sequences indicate a possible mechanism? Analysis of end-joining reactions of naked DNA introduced into mammalian cells by transfection led to the proposal of the slip-mispair-repair model (Roth et al., 1985; Roth and Wilson, 1986; Figure 6A). Terminal homologies, recognized upon partial unwinding, invite to pairing (a), followed by slip mispairing and primed DNA repair synthesis, which leads to a small sequence duplication (b). The resulting complex could be resolved either by repair against the lower strand, which results in a deletion (c), or against the upper strand, in which case the duplication is manifested (d). In the case of T-DNA end joining, this model would predict these duplications to arise at the position of the junction. Sequences in the CaMV genome close to the T-DNA borders were analyzed for the presence of different right T-DNA end–additional bases–left T-DNA end compositions (i.e. O □□□□ △). Surprisingly well fitting stretches were located various distances away from the T-DNA borders, and a few examples are listed in Figure 6B. Molecules rearranged along pathway c (Figure 6A) would be more difficult to find, since most of them would have represented nonviable deletions of essential regions of the replicon. This model could even apply to cases in which there are no extra bases at the end junction. The homologies to the two ends, which are recognized by the invading end, would not have been separated by some bases (i.e. O △ instead of O □□□□ △). In cases in which we find one, two, or three nucleotides, the source cannot be determined unambiguously. Such additions could also have been brought about by terminal deoxynucleotidyl transferase activity, which is known to be involved in mammalian immunoglobulin gene rearrangements (Alt and Baltimore, 1982; Akira et al., 1987).

Too few integrated T-DNA copies have been analyzed on the sequence level, and only a single study compares the integrated T-DNA and its junctions with the preintegration site (Gheysen et al., 1987). The pattern was shown to be rather complex, which might in part be due to rearrangements during the long maintenance of the tumor line in question. Small deletions and direct and inverted repeats that are duplications from nearby sequences have been found in several T-DNA–plant DNA junctions (Simpson et al., 1982; Holsters et al., 1983; Gheysen et al., 1987). One of our isolates contained, between the joined ends, a sequence of 8 nucleotides (Figure 5B, sequence y) which matched perfectly, but in inverse orientation, a sequence present in the (CaMV) T-DNA. This kind of duplication cannot be easily explained by the slip-mispair model. Small deletions and direct and inverted repeats have also been found in integration of adenoviral DNA into mammalian genomes (Doerfler et al., 1983; Lichtenberg et al., 1987). These phenomena therefore seem to be general attributes of site-unspecific integration in eukaryotes.

T-DNA molecules may enter the plant cell as single-stranded or double-stranded molecules, possibly protected at the 5′ end. A single-stranded form might have to be converted to a double-stranded form first and then integrate into plant genomic DNA via a mechanism as described above, or else it may invade the chromosomal DNA and only then become converted to a duplex.

Experimental Procedures

Bacterial Strains and Plasmids

E. coli NM522 (Gough et al., 1983) or HB101 was used for plasmid transformations. HB101(pRK2073) was used as a helper strain for bacterial conjugations (Ditta et al., 1980). The following Agrobacterium tumefaciens strains were used: LBA4404, a nontumorigenic octopine strain in which the Ti-plasmid has a complete deletion of the T-DNA (Hoekema et al., 1983), and GV3105, a wild-type oncogenic nopaline strain (Holsters et al., 1980). The following cloned CaMVs were used: pCa3, a SalI clone of CaMV-1841 in pUC19, with an ORF II of 480 bp (Gardner et al., 1981); pCa312, a SalI clone of Ca20-BalI in pUC19 with a mini ORF II of 29 bp; pCa355, identical to pCa312, except for a 5′ deletion of 17 bp removing the ORF II start codon and leaving a 12 bp intergenic region (both described by Brisson et al., 1984).

Construction of Plasmids

The kanamycin- and tetracyclin-resistant broad host range plasmid pRK252 (Bevan et al., 1984) was cut with SalI and XmaI to completion, and the 8 kb vector part was purified. A 68 bp double-stranded synthetic oligonucleotide-fragment consisting of, from 5′ to 3′, a SalI end, the nopaline C58 25 bp left border repeat with a point deletion in the conserved decanucleotide box, a unique XhoI site in the middle, the nopaline C58 25 bp right border repeat, and a 3′ XmaI end, was cloned in this modified pRK252 vector producing plasmid pEAP100. The SalI

site was removed by cutting with SalI, blunting with DNA–polymerase I (Klenow fragment, Biofinex), and religation (T4 DNA ligase, Biolabs), thereby creating a unique PvuI site, giving pEAP101. The CaMV strains have a unique SalI site in ORF V, and a unique XhoI site in ORF II. SalI clones in pUC19 of the respective CaMV mutant genomes were cut with XhoI, and cloned into XhoI-cut, dephosphatized (CIP, Boehringer) pEAP101, to give Km- and Ap-resistant intermediates. The pUC19 vector was removed by cutting with SalI, diluting, and self-ligation. This gave the plasmids pEAP104, 105, 112, 113; all about 15.6 kb in length (Figure 2).

The mutant left border was replaced by a wild-type C58 left border by restricting pEAP104 with PvuI and partially with XhoI, isolating the 15.6 kb fragment, and inserting into it a 47 bp double-stranded synthetic oligonucleotide consisting of, from 5′ to 3′, a PvuI end, a KpnI site, a 25 bp wild-type nopaline C58 border, a NotI site, and an XhoI end giving plasmid pEAP134. In pEAP134 the Ca355 part was exchanged by Ca3 in the XhoI site, yielding pEAP145.

Plasmid pCa355THB1 (10.3 kb) was constructed by cloning a 39 bp double-stranded synthetic oligonucleotide consisting of the nopaline C58-derived perfect 25 bp hybrid border (Koukolíková-Nicola et al., 1985) between XhoI linkers, into the XhoI site of pCa355 in such a way that the 5′ to 3′ border sequence is in the same orientation as the viral ORFs. The other orientation of the oligo gave pCa355THB2. pCa312-THB1 and pCa312THB2 were constructed in the same way. Restriction enzymes were purchased from Biolabs or Biofinex (BamHI and SalI).

Plant Inoculations

Three-week-old turnip plants (Brassica rapa variety Just Right) were inoculated by rubbing celite and either 25 μl of bacterial culture or 5 μg plasmid DNA on each of the two first true leaves. Tumors were induced by wounding at the crown with a toothpick dipped in a culture, as described by Grimsley et al. (1986). Work with Agrobacterial strains containing "pEAP constructs" as well as plant inoculations were done in a BL3 containment laboratory.

Viral DNA Isolation and Analysis

Systemically infected leaves were harvested 28 days post inoculation. Viral DNA was prepared as described by Gardner and Shepherd (1980). DNAs were restricted with BamHI, end-labeled with [α-^{32}P]dATP (Amersham) and DNA polymerase I (Klenow fragment), alcohol-precipitated, resuspended, further restricted with appropriate enzymes, and run out on a 8% polyacrylamide gel in 1× TBE (Tris-, Borate, EDTA) buffer. Gels were dried and exposed against X-ray films (Amersham, Hyperfilm-MP, RPM7). The viral DNA fragments to be sequenced were cloned as 1 kb BamHI–EcoRI fragments, and ligated directly from low melting agarose gels into plasmid pTZ18U (Mead et al., 1986). Sequencing was done directly from these plasmids, essentially as described by Zagursky et al. 1985), using an Amersham sequencing kit and CaMV-specific oligonucleotides.

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