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Genetics

Agrobacterium tumefaciens transfers single-stranded transferred DNA (T-DNA) into the plant cell nucleus

(β-glucuronidase/conjugation/extrachromosomal homologous recombination/transient expression)

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ABSTRACT **Transferred DNA (T-DNA) is transferred as a single-stranded derivative from *Agrobacterium* to the plant cell nucleus. This conclusion is drawn from experiments exploiting the different properties of single- and double-stranded DNA to perform extrachromosomal homologous recombination in plant cells. After transfer from *Agrobacterium* to plant cells, T-DNA molecules recombined much more efficiently if the homologous sequences were of opposite polarity than if they were of the same polarity. This observation reflects the properties of single-stranded DNA; single-stranded DNA molecules of opposite polarity can anneal directly, whereas single-stranded DNA molecules of the same polarity first have to become double stranded to anneal. Judging from the relative amounts of single- to double-stranded T-DNA derivatives undergoing recombination, we infer that the T-DNA derivatives enter the plant nucleus in their single-stranded form.**

The involvement of *Agrobacterium tumefaciens* in the formation of plant tumors, called crown galls, has been known since the beginning of the century (1). During the last 15 years, this area of research has been particularly productive, especially after the discovery that a defined piece of DNA, called transferred DNA (T-DNA), from the 200-kb Ti (tumor-inducing) plasmid of *Agrobacterium* is incorporated in the nuclear genome of plant tumor cells (2, 3). The T-DNA is delimited by two 25-bp imperfect direct repeats, called the right and the left borders. These repeats are the only elements required in cis for the transfer of the T-DNA to the plant cell. The DNA transfer is mediated by the gene products of the *vir* (virulence) genes, located on the Ti plasmid outside the T-DNA (for review, see refs. 4–7).

Only two Vir proteins, VirD1 and VirD2, are absolutely required for the processing of the T-DNA (ref. 8 and references therein). These two proteins recognize and nick the 25-bp-long border sequences. This results in tight attachment of VirD2 to a specific nucleotide on the 5′ end of the lower strand of T-DNA. This leads to the production of three types of T-DNA derivatives: circular double-stranded DNA (9–12), linear double-stranded DNA (13–15), and single-stranded DNA corresponding to the bottom strand of the T-DNA (16, 17). While the circular form is produced in *Agrobacterium* at low quantities upon stimulation by plant inducers and is considered to be a by-product of the T-DNA complex formation (12, 18), linear single- and double-stranded T-DNA derivatives are produced at much higher levels and at similar rates (refs. 14, 15, and 19; for review, see ref. 6). Both linear single- and double-stranded T-DNA derivatives have been found tightly attached to VirD2 protein (14, 15, 20–22). As the VirD2 protein was shown to pilot the T-DNA from the bacteria to the plant cell nucleus (23–27) and as it was suggested that VirD2 is also involved in integration (refs. 28 and 29; for review, see ref. 30), these two types of VirD2-linked T-DNA derivatives are good candidates for the T-DNA intermediate that is actually transferred to the plant cell nucleus. Generally, preference is given to the single-stranded structure (T-strand) model, for several reasons. (*i*) Strains missing the single-stranded DNA binding protein VirE2 can be complemented to virulence by coinoculation with a *virE2*-containing strain (31) or by using a *virE2* transgenic plant as transformation recipient (32). The involvement of a single-stranded DNA binding protein in the transfer can be used as a good argument for the single strandedness of the T-DNA. (*ii*) Since single-stranded derivatives have been detected in induced agrobacterial cells, the transfer mechanism has been compared to conjugation of the bacterial F plasmid (17, 33, 34). In the F conjugation system, the moving entity is a single-stranded DNA, although single-stranded molecules have never been detected in the donor cell (for review, see ref. 35). Whereas T-DNA-derived structures found in the bacterial cell do not necessarily represent transfer intermediates, free (i.e., unintegrated) T-DNA derivatives detected in the plant cells must correspond to the transfer intermediate or a derivative thereof. The high efficiencies of T-DNA-mediated transient expression (36, 37) and of intermolecular homologous recombination of T-DNAs containing sequence overlaps of the same polarity (38) and the finding of two T-DNAs integrated in a tail-to-tail configuration (39) point to the presence of free double-stranded T-DNA derivatives inside the plant cell nucleus.

We designed experiments that could detect the transient molecular properties of either a double- or a single-stranded DNA molecule. We concentrated on extrachromosomal homologous recombination that takes place early after entry into the nucleus (40) and can discriminate between single-stranded and double-stranded DNA molecules. The vast majority of extrachromosomal recombination events in plants can be described best by the single-strand annealing model (refs. 41 and 42; for review, see ref. 43), originally proposed for extrachromosomal recombination in mammalian cell nuclei (44, 45). Indeed, it could be directly demonstrated that single-stranded DNA is efficiently annealed in tobacco cells; cotransfection of single-stranded DNAs carrying overlapping nonfunctional parts of a marker gene resulted in higher intermolecular recombination efficiencies when the overlaps on both transfected molecules were of opposite polarity, in comparison to molecules with overlaps of the same polarity (46). The essential difference between the two types of recombining partners, therefore, is that DNA molecules of opposite polarity shorten the recombination pathway by direct self-annealing, whereas those of the same polarity first must become double-stranded.

The rationale for the work described in this report was to exploit known properties of extrachromosomal recombination to construct T-DNA molecules whose recombination

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Abbreviations: T-DNA, transferred DNA; GUS, β-glucuronidase.

behavior would directly indicate whether the transferred T-DNA consists of a single-stranded or double-stranded DNA molecule.

MATERIALS AND METHODS

Plasmids. The chimeric *uidA* gene from plasmid pGUS23 (47, 48) allows expression only in plant cells (27). The binary plasmid pLRG contains this entire *uidA* gene in the T-DNA and carries the bacterial gentamycin-resistance gene (27).

The recombination substrates, truncated elements derived from an *uidA* gene of pGUS23, were cloned into the binary plasmid pCGN1589, which carries the bacterial gentamycin-resistance gene (49). First, a *Bgl* II cassette carrying the *hpt* gene (hygromycin phosphotransferase) from the binary plasmid pGSC1614 (Plant Genetic Systems, Ghent, Belgium) was inserted into the *Bam*HI site of pCGN1589 resulting in the plasmid pCH. Into this plasmid, the nonfunctional deletion mutants of the *uidA* gene were cloned. The respective fragments of *uidA* were supplied with restriction sites appropriate for cloning, using PCR amplification. The N-terminal deletion mutant US (50) was then inserted between the *Xba* I and *Hin*dIII sites of pCH to result in pUS. The C-terminal deletion mutant that lacks 1245 bp of the 3′ end of the *uidA* gene and the terminator was cloned into the *Acc*56I site of pCH and pUS. In each case both orientations of the insert were obtained, resulting in the clones pGU, pU′G′, pGU.US, and pU′G′.US, as depicted in Fig. 2.

To test transfer efficiency of U′G′.US and GU.US to plant cells, a *Hin*dIII cassette containing a *bar* gene (coding for the phosphinotricin acetyltransferase) was cloned from pCIB4232 (CIBA–Geigy), inside the T-DNA, close to the left border of pU′G′.US and pGU.US. In both constructs, the 3′ end of the *bar* gene faces the left border. All plasmids were introduced into *Agrobacterium* strains by electroporation (51).

***Agrobacterium* Strains.** The following strains were used: GV3101(pPM6000), a cured nopaline strain that contains the disarmed octopine plasmid pPM6000 (52); UIA143(pSVB125), a nopaline *recA*$^-$ strain (53); and GV3101(pPM6000K), a derivative of GV3101(pPM6000) that was rendered transfer-defective due to a large deletion in the *virD2* gene (27).

Seedling Assay. *β-Glucuronidase (GUS) assays.* The seedling assay was performed as described (54). In all experiments the transfer-defective strain GV3101(pPM6000K, pLRG) was used to monitor any T-DNA-transfer-independent GUS activity. The incubation time of the seedlings in the 5-bromo-4-chloro-3-indolyl glucuronide solution was chosen such that a range of 300 spots per 100 plants for the substrate(s) containing the overlaps in opposite orientation was achieved (2 or 3 days and 10–15 days for *recA*$^+$ and *recA*$^-$ strains, respectively).

Integration assay. The procedure as described above was applied for the transformation assay, except that after cocultivation the seedlings were washed with 10 mM $MgSO_4$ and placed on MS medium containing naphthylacetic acid (0.1 mg/liter), benzylamino purine (1.0 mg/liter), glufosinate ammonium (20 mg/liter, purchased from Riedel-de-Haën), claforan (500 mg/liter), and vancomycin (500 mg/liter). Calli were counted after 3–4 weeks.

Transfection with PEG. *Nicotiana plumbaginifolia* protoplasts were transfected using 15 μg of plasmid DNA per 10^6 protoplasts and PEG, as described (48).

RESULTS

We constructed two sets of T-DNAs. The T-DNAs of the first set contain homologous sequences in opposite orientation. Annealing of the repeated sequences is expected to occur directly from single-stranded T-DNA intermediates or, after exonucleolytic degradation, from double-stranded T-DNA intermediates (Fig. 1). T-DNAs of the other set contain the same homologous sequences, but in the same orientation. A single-stranded T-DNA intermediate cannot anneal, whereas a double-stranded T-DNA intermediate of this set is expected to yield annealing competent fragments after nucleolytic degradation and concomitant exposure of the complementary repeat in the single-stranded form. If the transferred T-DNA is double-stranded, both sets should show the same or similar recombination efficiencies. A single-stranded DNA molecule would recombine with drastically different efficiencies, depending on the relative orientation of the sequence repeat (see Fig. 1).

The recombination efficiencies were quantified in a well-established transient assay system (48, 55), using a modified GUS (*uidA*) gene that is only expressed in plant cells and not in bacteria (54).

Five binary vectors containing truncated *uidA* elements in their T-DNA were designed (Fig. 2). (*i*) Plasmid pUS contains the 3′ part of a truncated *uidA* gene (recombination substrate, US) that is oriented with the polyadenylylation signal facing the T-DNA left border. (*ii*) Plasmid pGU contains the 5′ part of *uidA* (recombination substrate, GU) with an overlap of 557 nt with the *uidA* part of pUS, the promoter facing the T-DNA right border. (*iii*) Plasmid pU′G′ contains the same *uidA* part as pGU but in the opposite orientation with respect to the border repeats (recombination substrate, U′G′). (*iv*) Plasmid pGU.US contains the combination of the *uidA* elements from

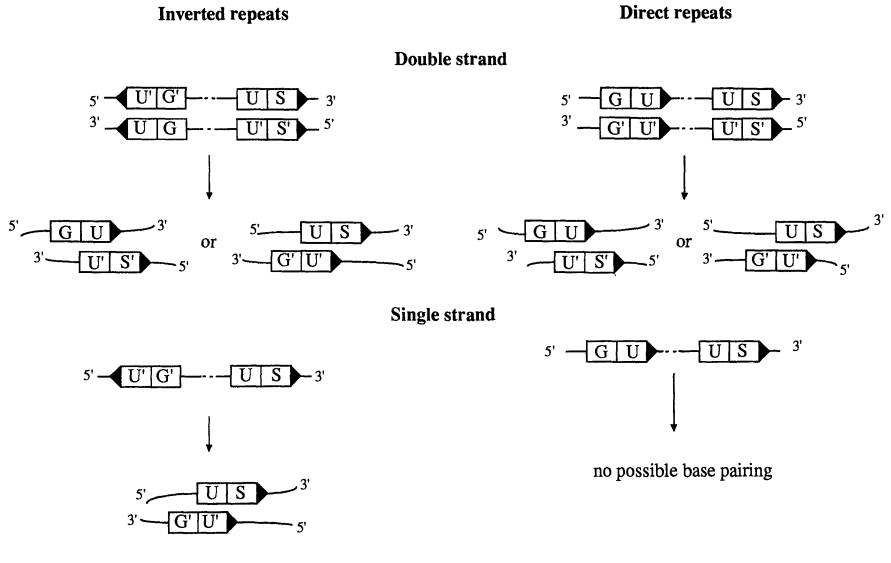

FIG. 1. Annealing and recombination behavior of single- and double-stranded DNA molecules carrying repeated sequences of direct or indirect orientation. Recombination substrates in which the homologous sequences (U and U′, for both strands) are supplied, in cis or in trans (dotted lines), in opposite orientation, can anneal as single-stranded or double-stranded molecules. In contrast, recombination partners in which the homologous sequences are provided in the same orientation can anneal starting from the double-stranded configuration only. G, U, S, 5′, central, and 3′ parts of the GUS gene, respectively. Solid triangles show the direction of the gene.

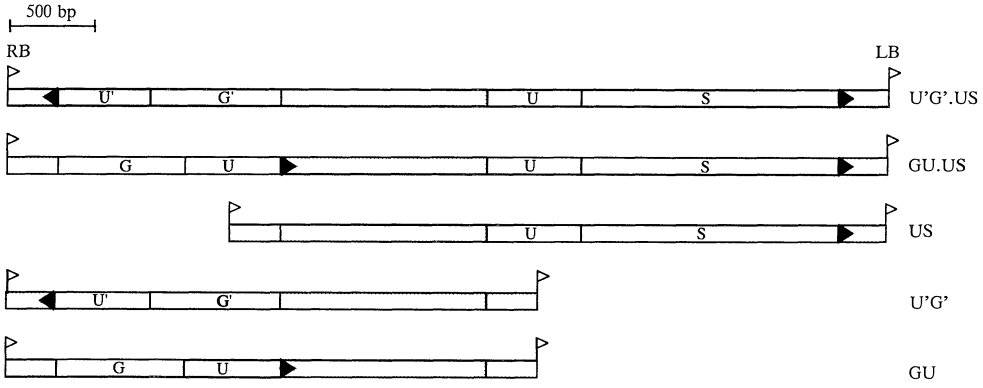

FIG. 2. Recombination substrates present in the T-DNA of the plasmids pU'G'.US, pGU.US, pUS, pU'G', and pGU. Contrary to convention the right border (RB) is marked on the left side. This allows us to show the "T-DNA bottom strand" (here upper strand) in the otherwise conventional 5' → 3' direction and to make this figure parallel with Figs. 1 and 3. Grey areas represent the *hptII* gene and the T-DNA part of plasmid pCGN1589. Symbols are as in Fig. 1.

pUS and pGU separated by a hygromycin-resistance gene (recombination substrate, GU.US). (*v*) pU'G'.US contains a combination of the *uidA* elements from pUS and pU'G' (recombination substrate, U'G'.US), each element separated by a hygromycin-resistance gene. In addition, the binary plasmid pLRG containing a functional *uidA* gene was used to monitor the transfer efficiency (for diagrams of plasmids, see Fig. 2).

T-DNAs Carrying Inverted Repeats Recombine More Efficiently Than the T-DNAs Carrying Direct Repeats. Tobacco seedlings were cocultivated with the *Agrobacterium* GV3101-(pPM6000, pGU.US), GV3101(pPM6000, pU'G'.US), GV3101(pPM6000, pLRG), or GV3101(pPM6000K, pLRG). The latter strain is transfer-deficient due to a deletion in the *virD2* gene (27). Homologous recombination events restoring the *uidA* gene were monitored by assaying for GUS activity. The GUS activity was measured either fluorometrically after incubation of the seedling extracts with 4-methylumbelliferyl β-D-glucuronide as substrate or by counting blue spots after incubation of the seedlings with 5-bromo-4-chloro-3-indolyl glucuronide. In three experiments, and for both types of assay, the construct pU'G'.US gave GUS values one order of magnitude higher than values produced by the construct pGU.US (Table 1). In the same experiments, the GUS activity measured after transfer of the intact *uidA* gene was 100–150 times higher than the one measured for the U'G'.US construct. Thus, the T-DNA substrate that contains the homologies in opposite polarity restored the *uidA* gene more efficiently than did the construct with homologies in direct orientation. This strongly suggests that single-stranded DNA serves directly as substrate for recombination.

However, we needed several control experiments to exclude the possibility that the results observed may be the consequence of events other than annealing and homologous recombination in the plant cell. Such events or facts could be (*i*) recombination taking place within bacteria, (*ii*) a difference in the efficiency of transfer to the plant cell between the constructs, or (*iii*) a difference in the recombination efficiency, in plant cells, of the two double-stranded substrates.

Recombination Events Within the Bacteria Cannot Account for the Recombination Behaviors of T-DNAs Carrying Direct or Indirect Repeats. (*i*) The recombination substrates were tested in a *recA*$^-$ *Agrobacterium* strain to exclude intraplasmid recombination within the bacteria. The substrates pU'G'.US, pGU.US, and the control plasmid pLRG were introduced into the *recA*$^-$ *Agrobacterium* UIA143 (pSVB125) and assayed, after infection, for recombination in the plant cells. The *recA*$^-$ strain UIA143(pSVB125, pLRG) proved to be much less efficient in T-DNA transfer than the *recA*$^+$ proficient control strain GV3101(pPM6000, pLRG) used earlier. Therefore, only the sensitive histochemical assay was used for analysis of this experiment. In two experiments, the substrate U'G'.US was one order of magnitude more efficient than the substrate GU.US, in performing homologous recombination (see Table 1). These results are similar to the ones obtained using the *recA*$^+$ strain GV3101(pPM6000). Thus, recombination of the double-stranded plasmid pU'G'.US inside the bacteria cannot explain the difference that was observed between the recombination behavior of U'G'.US and GU.US.

(*ii*) Single-stranded annealing and recombination of the T-DNA intermediate could also occur in the bacteria, as a *recA*-independent process. Our constructs have been designed so that such an event would give rise to a molecule unable to be transferred into the plant cell. Homologous recombination via single-strand annealing in the bacteria would free the VirD2 pilot protein, which is essential for the T-DNA transfer, from the T-DNA intermediate processed from pU'G'.US (and pU'G') (Fig. 3). Thus, the recombination events as revealed by the GUS activity did occur in the plant cell.

U'G'.US and GU.US Are Transferred to the Plant Cell Nucleus with the Same Efficiency. To test whether each substrate is transferred to the plant cell nucleus with the same efficiency, a *bar* gene (encoding phosphinothricin acetyltransferase) was cloned into the T-DNA of pU'G'.US and pGU.US, and these T-DNAs were then tested for their ability to induce calli resistant to glufosinate ammonium and, in parallel, for their recombination behavior. Both constructs behaved identically in the stable transformation assay, whereas measurement of the GUS activity showed the same difference as between U'G'.US and GU.US (Table 1). Therefore, U'G'.US and GU.US are transferred with similar efficiencies to the plant cell nucleus.

The Double-Stranded Recombination Substrates of the Same Polarity and of Opposite Polarity Recombine with the Same Frequency in the Plant Cell. The double-stranded plasmids pU'G'.US and pGU.US were transfected into *N. plumbagin-*

Table 1. Test of the intramolecular and intermolecular recombination substrates

Assay	Substrates	*recA*$^+$ MUG	*recA*$^+$ X-Glu	*recA*$^-$ X-Glu	PEG transfection MUG	Transformation, no. of calli
Intramolecular	pU'G'.US/pGU.US	11.2 ± 0.2 (3)	10.2 ± 0.7 (3)	13.2 ± 4 (2)	1.5 ± 0.4 (5)	0.81 ± 0.12 (4)
Intermolecular	(pUS+pU'G')/(pUS+pGU)		16 ± 3.8 (4)		0.9 ± 0.2 (5)	

Normalized values of the indicated number of experiments (in parentheses) (average ± SD) are given. *recA*$^+$ corresponds to the *Agrobacterium* strain derived from GV3101, and *recA*$^-$ corresponds to the strains derived from UIA143. MUG, 4-methylumbelliferyl β-D-glucuronide; X-Glu, 5-bromo-4-chloro-3-indolyl glucuronide.

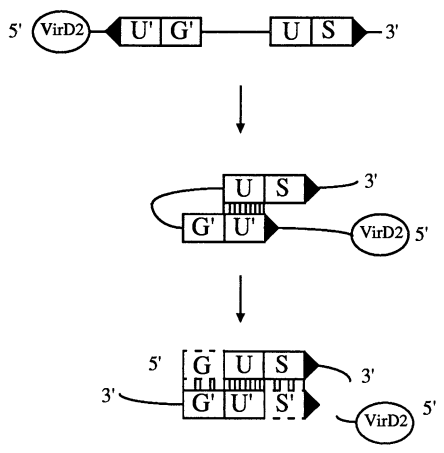

FIG. 3. Single-strand annealing of a putative T-strand of an intramolecular recombination substrate inside an *Agrobacterium* cell. The restoration of the *uidA* gene (GUS) is coupled to the loss of the VirD2 protein. Symbols are as in Fig. 1.

ifolia protoplasts, using the PEG procedure. Efficiencies of homologous recombination were assayed fluorometrically (as described in ref. 48). No significant difference was found between the resulting GUS activities (Table 1). Thus, the difference in recombination of the recombination substrates transferred by *Agrobacterium* cannot be explained by a difference in recombination potential of the double-stranded forms.

Intermolecular Recombination. In pU'G'.US and pGU.US, the recombination substrates are on the same T-DNA unit (Fig. 2). Homologous recombination can thus occur either intramolecularly (within the same T-DNA) or intermolecularly (between two T-DNAs). To test for intermolecular recombination events, the recombination substrates were placed on different T-DNAs. The plasmids containing the isolated recombination substrates US, U'G', and GU were introduced separately into different agrobacteria for analysis of the frequency of the intermolecular recombination events of the pairs (US, GU) and (US, U'G'). [The corresponding plasmid pairs were also cotransfected into protoplasts and intermolecular recombination was assayed fluorometrically. The results showed that both pairs of double-stranded plasmids recombined with the same efficiency (Table 1)].

The infection of tobacco seedlings was performed using suspensions containing, in a 1:1 ratio, the strain GV3101-(pPM6000, pUS) combined either with GV3101(pPM6000, pGU) or with GV3101(pPM6000, pU'G'). Because of the low number of events expected, recombination was analyzed only by the histochemical assay. In four experiments, the intermolecular homologous recombination between the substrates of opposite polarity, US and U'G', was found to be more than one order of magnitude higher than between the substrates of the same polarity, US and GU (Table 1). To check whether conjugation events may allow the two recombination substrates to meet in the same *Agrobacterium* cell prior to the transfer to the plant cell, the T-DNA transfer-defective strain 3101(pPM6000K, pLRG) was cocultivated with tobacco seedlings in the presence and absence of the transfer-proficient but pLRG-lacking strain 3101(pPM6000). In neither case was GUS activity detected, arguing against mobilization of pLRG, and any of its derivatives, into the transfer-proficient strain. Thus, the recombination substrates have to meet in the plant cell.

The fact that we can reproduce our previous finding with the recombination substrates in trans is the final proof that (*i*) the detected effect is not due to intrabacterial recombination and (*ii*) the effect is not specific for intramolecular recombination events in plants. Thus, this result strengthens our conclusion that single-stranded DNA is transferred from *Agrobacterium* to the plant cell nucleus.

DISCUSSION

It has been suggested that the T-DNA transfer mechanism was derived from bacterial conjugation (17, 33, 34, 56) although parts of the transfer have also been compared to steps in viral infection (34, 57). Sequence comparison of *Agrobacterium* virulence and IncP plasmid transfer genes has revealed homology (58). In addition, the transfer origin *nic* of RP4 and border sequences of various *Agrobacterium* strains have a strong core homology (59). The IncP system differs in many aspects from F type conjugation (60) and the nature of the T-DNA in the IncP type conjugation has never been established.

By using extrachromosomal recombination in plant cells, we could show that T-DNA substrates that are able to anneal in cis or in trans in their single-stranded forms exhibited recombination efficiencies one order of magnitude higher than substrates that could anneal only as a double-stranded molecule. These results demonstrate that single-stranded DNA molecules enter into the plant cell nucleus.

Naked single-stranded molecules are efficiently converted into double strands (61), and as such they allow recombination of homologous segments of the same polarity. This type of recombination has been described for molecules entering plant cells via direct gene transfer (46). We therefore think that there is no need to invoke double-stranded DNA molecules entering into the plant cell nucleus and to consider two types of transfer. Thus, we propose that the identified single-stranded T-DNA derivative is the unique T-DNA intermediate entering the plant cell nucleus.

Our data raise the question whether single- or double-stranded T-DNA derivatives integrate into the nuclear genome. Reports describing direct gene transfer into plant cell protoplasts revealed a similar efficiency of integration of single- and double-stranded linear DNA (46, 61). As far as the T-DNA intermediates can be compared to naked DNA, these data favor the integration of single-stranded T-DNA intermediates into the plant nuclear DNA, because of the higher amount of single-stranded form in the nucleus. However, also double-stranded T-DNA derivatives may play a role in the natural transformation process, allowing transient overexpression of certain T-DNA genes responsible for tumor formation and thus increasing the competence of the plant for the integration of exogenous DNA (62).

The finding that a single-stranded DNA molecule carries the genetic information from *Agrobacterium* to plants further supports the idea that this DNA molecule interacts with the VirE2 protein (63). As mentioned above, transfer of a single strand by conjugation has only been shown for F-plasmid type conjugation. This report extends this finding to a very particular type of conjugation, a conjugation occurring between a prokaryote, *Agrobacterium*, and a higher eukaryote, the plant. Sequence comparison and functional studies have shown that this conjugation process is related to IncP type conjugation (58, 64, 65). The VirD2 protein, which is the equivalent to the TraI protein of the IncP conjugation system, has evolved to carry the T-DNA intermediate to the plant cell nucleus (23–27) and possibly to help in integration (refs. 28 and 29; for review, see ref. 30). It will be interesting to see how other IncP type Tra functions may have evolved to allow efficient transformation of the eukaryotic cell.

Note Added in Proof. After submission of this manuscript, a paper was published demonstrating association of single-stranded T-DNA with tobacco cells (66).

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