The EMBO Journal Vol.2 No.12 pp.2143–2150, 1983

Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity

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Communicated by M. Van Montagu
Received on 22 July 1983

A Ti plasmid mutant was constructed in which all the oncogenic functions of the T-DNA have been deleted and replaced by pBR322. This Ti plasmid, pGV3850, still mediates efficient transfer and stabilization of its truncated T-DNA into infected plant cells. Moreover, integration and expression of this minimal T-DNA in plant cells does not interfere with normal plant cell differentiation. A DNA fragment cloned in a pBR vector can be inserted in the pGV3850 T-region upon a single recombination event through the pBR322 region of pGV3850 producing a co-integrate useful for the transformation of plant cells. Based upon these properties, pGV3850 is proposed as an extremely versatile vector for the introduction of any DNA of interest into plant cells.
Key words: Ti plasmid/vector/plant cell transformation

Introduction

The Ti plasmid of *Agrobacterium tumefaciens* has long been recognized as a natural vector for the transfer of DNA to plant cells. *Agrobacterium* harbouring a Ti plasmid causes neoplastic transformation, called crown gall, of the wounded tissue of a wide range of dicotyledonous plants (for reviews, see Nester and Kosuge, 1981; Bevan and Chilton, 1982; Kahl and Schell, 1982; Zambryski *et al.*, 1983). Crown gall tissues synthesize novel metabolites called opines, and the Ti plasmids are classified according to the type of opine they specify. The most commonly used Ti plasmids are those which code for the metabolism of nopaline or octopine. The molecular basis of crown gall transformation is the transfer and stable integration of a well-defined T-DNA (transferred DNA) fragment of the Ti plasmid into the plant cell genome. The mechanism of DNA transfer as well as the functions encoded by this DNA have only begun to be understood. However, we can already begin to take advantage of this system to modify the Ti plasmid for application to the genetic engineering of plants.

Knowledge of the T-DNA sequences which define the borders of the transferred DNA is a basic requirement for the use of the Ti plasmid as a vector for DNA transfer to plant cells. The borders of the T-DNAs in nopaline (Lemmers *et al.*, 1980; Yadav *et al.*, 1980,1982; Zambryski *et al.*, 1980,1982) and octopine (Thomashow *et al.*, 1980a,1980b; De Beuckeleer *et al.*, 1981; Simpson *et al.*, 1982; Holsters *et al.*, 1983) tumor lines have been accurately determined. In octopine tumors a 13.6-kb TL-DNA is always present, and an additional 6–7 kb TR-DNA derived from an adjacent region of the Ti plasmid is sometimes also present. The nopaline T-DNA is a continuous stretch, ~23 kb of the entire 200-kb plasmid. The ends of this T-DNA as found in the plant genome are very precise; for example, the right end of the T-DNA junction of four borders varies over only 1 bp (Zambryski *et al.*, 1982), and the left junction of four borders varies over 90 bp (Zambryski *et al.*, 1982; Yadav *et al.*, 1982). The precision of the T-DNA integration allows the insertion of foreign DNA within the borders of the T-DNA and ensures its transfer to the plant cell genome (Hernalsteens *et al.*, 1980; Holsters *et al.*, 1982).

The second most important criterion, if one expects to utilize the Ti plasmid as a vector system, is that the transformed plant cells can differentiate in a normal manner rather than grow in a tumorous fashion. To obtain normal differentiating cells after T-DNA transfer requires knowledge of the functions encoded by the T-DNA which prevent this normal differentiation. Thus, the genes coding for these functions have been localized to specific regions of the T-DNAs of both octopine and nopaline Ti plasmids by intense genetic analysis (Holsters *et al.*, 1980; Garfinkel and Nester, 1980; Garfinkel *et al.*, 1981; Ooms *et al.*, 1980,1981; De Greve *et al.*, 1981; Leemans *et al.*, 1982; Willmitzer *et al.*, 1982,1983; Joos *et al.*, 1983; Ream *et al.*, 1983).

One of the results of these genetic studies was the isolation of a class of mutants (*shi*) which induced shoots containing either untransformed plant cells or cells transformed with a shortened T-DNA segment which no longer contained any oncogenic functions. The latter transformed shoots regenerated into normal and fertile plants which even transmitted T-DNA-specific sequences through meiosis as a single dominant Mendelian factor (Otten *et al.*, 1981; De Greve *et al.*, 1982). While these results were important in establishing that normal and fertile plants could be produced following Ti plasmid-mediated transformation, there is an aspect of uncertainty in these studies as there is no information about how the 'transformed' shoots containing the shortened T-DNA occur. Either a T-DNA deletion has occurred subsequent to T-DNA integration, or there has been an independent insertion of a shorter T-DNA segment. Furthermore, the isolation of these 'transformed' shoots is a low frequency event (1/300).

Another class of Ti plasmids (*roi*) which induce root formation have been shown recently to also transform plant cells and allow normal plant differentiation (Barton *et al.*, 1983); and in this case, the entire T-DNA was even transmitted to progeny plants. However, it is not known why this particular T-DNA does not interfere with regeneration and whether or not its non-oncogenic phenotype in infected plant cells will be reproducible in all conditions of plant cell growth.

Based on these findings, we tried systematically to design a modified T-region of the Ti plasmid which should have the following properties: (i) T-DNA border regions; (ii) no genes which prevent normal differentiation of transformed plant cells; and (iii) a genetic marker to monitor the presence of the T-DNA. Using the nopaline Ti plasmid (pTiC58), we have constructed a deletion which removes all the T-DNA except

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for the borders and which also contains the nopaline synthase gene, a T-DNA-specific marker. In addition, this Ti plasmid contains the widely used cloning vehicle pBR322 between the borders of the mutated T-DNA: this makes this Ti plasmid an extremely versatile acceptor for the introduction of any foreign gene contained in a pBR-like plasmid. The construction of this Ti plasmid vector and its properties are described below.

Results

Construction of Ti plasmid vector pGV3850

Figure 1 shows the construction of the T-DNA deletion mutant pGV3850. This was possible due to the existence of a clone pAcgB which only contained the left and right border regions of the nopaline T-DNA. This clone has been previously described (Zambryski *et al.*, 1980,1982); it was obtained by reisolating portions of the T-DNA from transformed tobacco DNA. pAcgB contains the junction of two T-DNA copies which are arranged in tandem so that it contains the left and right borders of the T-DNA. In addition, pAcgB contains the nopaline synthase gene which maps immediately adjacent to the right T-DNA border (Holsters *et al.*, 1980; Depicker *et al.*, 1982). An interesting application of this clone is to use it to create a deletion of the internal oncogenic portion of the T-DNA.

To select for the genetic events which lead to the formation of the T-DNA deletion, we used an acceptor Ti plasmid pGV3839 (Joos *et al.*, 1983) which contains the kanamycin-resistance (Km^R) gene near the centre of its T-DNA (Figure 1A). Thus, pAcgB which carries a ColE1-specific *bom* site in its pBR322 portion is directly mobilized to an *Agrobacterium* strain containing pGV3839 using helper plasmids as described recently (Van Haute *et al.*, 1983). The ampicillin resistance (Ap^R) of pBR322 is used to select for the first single cross-over event with the nopaline Ti plasmid. As pBR322 cannot replicate in *Agrobacterium*, the only way that the ampicillin resistance can be stabilized in *Agrobacterium* is by a cross-over event upon homologous recombination with pGV3839 through one of the homology regions near the T-DNA borders. By a second cross-over event through the other homology region at the other border, the central portion of the T-DNA of pGV3839 including the Km^R gene is replaced by the pBR322 sequences of the clone pAcgB. The DNA molecule containing the central portion of the T-DNA including the Km^R marker is lost as it cannot replicate. Second recombinants are thus ampicillin-resistant and kanamycin-sensitive (Figure 1B). These recombination events were verified by restriction enzyme analysis and Southern blot hybridization (data not shown), and the detailed restriction map of the modified T-DNA of pGV3850 is shown in Figure 1C.

Phenotype of pGV3850 after infection of plant tissue

We tested whether *Agrobacterium* carrying pGV3850 could still transfer its T-DNA to plant cells in the absence of the internal T-DNA functions. All the DNA which encodes RNA transcripts involved in the production of the undifferentiated phenotype as well as additional sequences which encode transcripts of as yet unknown function (Joos *et al.*, 1983; Willmitzer *et al.*, 1983) are deleted in pGV3850. Only the DNA encoding nopaline synthase (*nos*) and a transcript of unknown function which maps in *Hin*dIII fragment 10 remain. While it has been shown that removing one or more transcripts does not interfere with T-DNA transfer, an

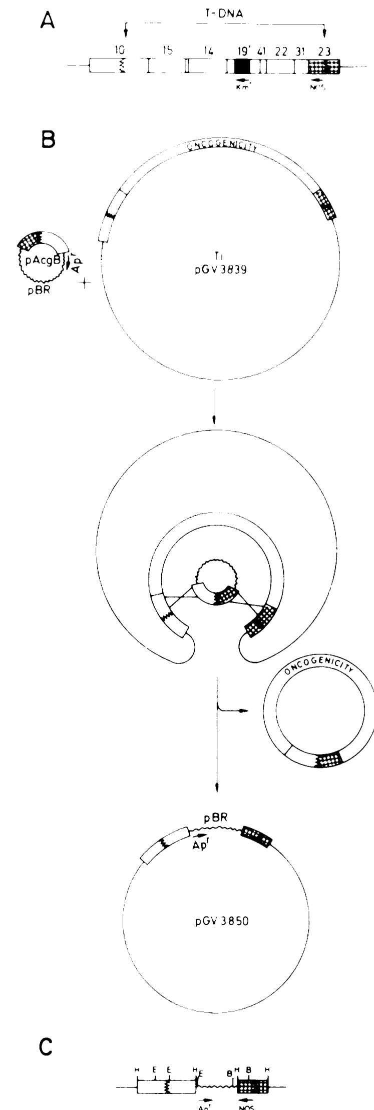

Fig. 1. Construction of Ti plasmid vector pGV3850. **(A)** The T-DNA of nopaline Ti plasmid pGV3839 (Joos *et al.*, 1983). The numbers refer to the *Hin*dIII fragments of the C58 nopaline Ti plasmid (Depicker *et al.*, 1980) and the vertical bars represent the *Hin*dIII restriction sites. One of the *Hin*dIII fragments labelled 19′ (normally fragment 19 in the wild-type map) contains a substitution of the nopaline Ti plasmid *Sma*I fragment 24 (Depicker *et al.*, 1980) for the *Hin*dII fragment of pKC7 (Rao and Rogers, 1979) which contains the Km^R gene: this deletion substitution is indicated by the black area. The two *Hin*dIII fragments 10 and 23 contain the T-DNA borders indicated by the jagged lines. In addition, the map position of the *nos* gene in *Hin*dIII fragment 23 is indicated, sequences homologous to *Hin*dIII-23 are indicated by the stippled regions throughout the figure. **(B)** The double cross-over events between pGV3839 and pAcgB leading to the formation of pGV3850. pAcgB contains only a portion of the left and right *Hin*dIII T-DNA border fragments 10 and 23 joined together at the T-DNA borders as indicated by the jagged line; these DNA sequences were cloned as a *Hin*dIII fragment in pBR322 as described (Zambryski *et al.* 1980,1982). The pBR322 sequences are indicated as a wavy line. The orientation of the DNA sequences in pAcgB and in pGV3839 are from left to right as they normally occur in the Ti plasmid. The T-DNA portion internal to the *Hin*dIII border fragments 10 and 23 is indicated as 'oncogenicity'. The double cross-over event results in the production of pGV3850 and a DNA circle containing the T-DNA of pGV3839 which is lost as it is not a replicon. Again, the T-DNA borders are indicated by jagged lines. The orientation of the two border fragments in pAcgB is: left border fragment 10 – pBR322 – right fragment 23; this results in the deletion of the internal portion of the T-DNA region and its replacement with pBR322. **(C)** A detailed restriction map of the modified T-DNA of pGV3850. The abbrevations used are: Ap^r, ampicillin resistance; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; NOS, nopaline synthase.

analysis of the effect of a complete deletion of the internal portion of the nopaline T-DNA had not yet been tested. We assayed for the presence of nopaline in pGV3850-infected plant tissues as evidence that T-DNA transfer had occurred. Potato and carrot discs, as well as tobacco and petunia plantlets inoculated with *Agrobacterium* containing pGV3850 all produced nopaline-positive tissue (data not shown). These results agree with previous ones using large T-DNA deletions of both the nopaline and octopine Ti plasmids which also produce opine-positive tissue (Leemans *et al.,* 1982; Joos *et al.*, 1983). They provide conclusive evidence that a deletion of the entire internal portion of the nopaline T-DNA does not affect the transfer of the remaining border regions of the mutant T-DNA. Furthermore, all these data suggest that it is unlikely that the T-DNA itself encodes products involved in T-DNA transfer, integration and stabilization; these functions are likely encoded elsewhere, either by the Ti plasmid, other *Agrobacterium* DNA, or by the plant genome.

Nopaline-positive carrot and potato tissues were obtained when the discs were incubated on solid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) in the presence of growth-regulating substances, either auxin alone [naphthalene acetic acid (NAA) (1 mg/l), or auxin and cytokinin (NAA, 1 mg/l; benzylaminopurine (BAP), 0.2 mg/l]. Without added hormones, there is no response of the potato disc to infection with pGV3850. However, small callus growths are produced when the disc is infected with pGV3850 in the presence of hormones. This callus tissue is nopaline-positive. It is interesting that the callus growth in the presence of hormones is in part due to the presence of the Ti plasmid as this response does not occur with an *Agrobacterium* strain cured of its Ti plasmid. This is evidence that there may be regions outside the T-DNA encoding products which affect plant cell growth.

Nopaline-positive tobacco and petunia tissues were also obtained by analyzing the response of decapitated plantlets to infection with *Agrobacterium* harbouring pGV3850. Tumors are not produced as expected; however, tiny calli are observed at the wound site 4 – 8 weeks after infection. This latter effect is never seen when *Agrobacterium* cured of its Ti plasmid is used to infect plants; thus, this response may be due to some functions outside the T-DNA region of the Ti plasmid as discussed above for the potato response. This slight though significant response to pGV3850 allowed us to obtain transformed tissue and to further analyze the phenotype when the T-DNA of pGV3850 is stably integrated into the plant cell genome.

Regeneration of normal plants containing the non-oncogenic T-DNA of pGV3850

As pGV3850-transformed plant tissues are not tumorous in their growth pattern, we tested whether these transformed cells could regenerate whole plants which still contain the minimal T-DNA of pGV3850. This result would be particularly useful since foreign DNAs of interest could be inserted within the T-DNA of pGV3850 in order to transform plant cells, and subsequently produce normal plants containing these foreign DNAs. Tobacco plantlets infected with pGV3850 were used as a model system.

Four to eight weeks after infection of decapitated tobacco stems with *Agrobacterium* carrying pGV3850, tiny calli develop at the wound surface. This wound surface is removed from the plantlet and incubated further on Linsmaier and Skoog (LS) medium (Linsmaier and Skoog, 1965) containing

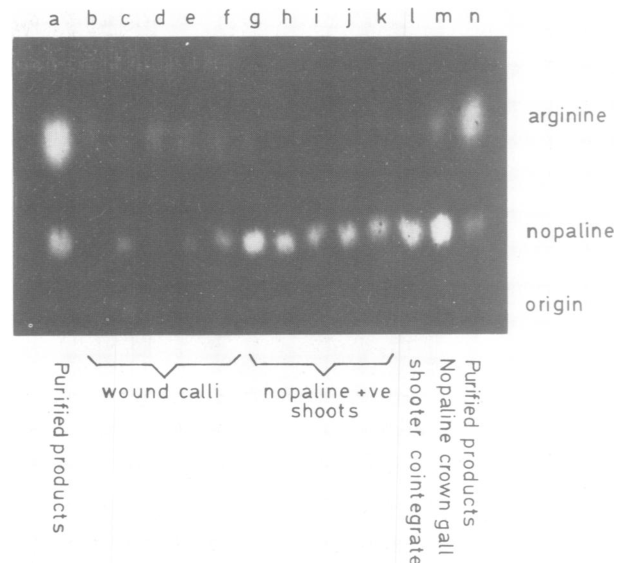

Fig. 2. Nopaline tests of pGV3850-infected tobacco tissues. Results of nopaline tests are shown of plant tissues isolated upon infection with the *Agrobacterium* strain C58C1 (pGV3850). The samples (also 2 μl) in **a** and **n** were derived from a control solution containing arginine and nopaline each at 0.5 mg/ml. Samples **b – f** are derived from callus grown on LS medium (Linsmaier and Skoog, 1965) with auxin (NAA, 1 mg/l) and cytokinin (BAP, 0.2 mg/l) which was derived from the wound surface of decapitated tobacco plantlets infected with *Agrobacterium* containing pGV3850. Samples **g – k** were derived from differentiating shoots obtained after nopaline-positive pGV3850-infected wound callus was placed on LS regeneration medium containing 1 mg/l BAP. Sample **l** is from callus tissue growing on hormone-free medium obtained from infection with *Agrobacterium* carrying a co-integrate between pGV3850 and the oncogenic genes of the octopine TL-DNA (those contained on *Eco*RI fragment 7 (De Vos *et al.*, 1981) of the octopine B6S3 Ti plasmid) which were cloned in a plasmid containing pBR322 sequences for homologous recombination with pGV3850 (J. Leemans, R. Deblaere, unpublished results). Sample **m** was derived from tumor callus produced by infection with *Agrobacterium* containing wild-type Ti plasmid C58.

auxin (NAA, 1 mg/l) and cytokinin (BAP, 0.2 mg/l). After further growth, a portion of the growing callus was tested for *nos* activity and a clear, but weak response was obtained (Figure 2d). This result is probably due to the presence of both transformed and untransformed cells in the callus tissue since there is no selection for transformed cells. A portion of this *nos*-positive tissue was transferred to shoot regeneration medium (LS medium plus BAP, 1 mg/ml). Subsequently, the shoots (Figure 3A) were transferred to hormone-free medium; all grew in a normal fashion and developed roots. Finally, the shoots were tested for *nos* activity: 11 out of 130 were positive for nopaline (see for example, Figure 2g, h, i, j, k). All 11 showed a strong *nos* signal indicating that the shoots are probably composed entirely of transformed cells. These *nos*-positive shoots have been grown further into whole plants and one is displayed in Figure 3B.

The *nos* activity is a useful marker to assay for the presence of pGV3850-transformed cells. Figure 2 shows the *nos* tests for the tissue shown in Figure 3, and also additional pGV3850-transformed tissues. For example, Figure 2b, c, d, e and f show the amount of nopaline present in various primary calli obtained from the wound surface of different tobacco plantlets and which were subsequently grown on solid agar medium containing auxin and cytokinin. These different calli produce different amounts of nopaline, from zero (Figure 2b) to significant amounts (Figure 2f). Furthermore,

Fig. 3. Regeneration of whole plants containing the shortened T-DNA of pGV3850. (**A**) The appearance of normal shoots which begin to form 4 weeks after nopaline-positive wound callus (obtained following infection of tobacco stems with *Agrobacterium* containing pGV3850) is placed on regeneration medium (LS medium plus 1 mg/l BAP). When these shoots are 1 cm long they are removed from the primary callus and placed on LS medium without hormones to allow the formation of roots. When the shoots have grown sufficiently to allow a portion to be removed, a nopaline test is performed. *Nos*-positive shoots are removed to larger pots to allow further growth of the transformed plants. A *nos*-positive pGV3850-transformed plant is shown in **B**.

the amount of nopaline present in the original callus is directly related to the frequency of occurrence of *nos*-positive shoots when the calli are placed on regeneration medium. For example, the calli in Figure 2d and 2f gave rise to *nos*-positive shoots at a frequency of 9 and 78%, respectively. However, not all inoculated plantlets produce *nos*-positive wound callus. In three separate experiments we have found that only 25% of the plants respond: in the first experiment 1 in 4, in the second experiment 2 in 8, and in the third 2 in 7. Nevertheless, even at this lowered frequency it is very easy to detect

transformed tissue by a simple test for the presence of nopaline.

We have regenerated >50 plantlets from several independent infection experiments; in all cases the plantlets appear completely normal as in the sample shown in Figure 3B. In addition, these regenerated plants respond as wild-type tobacco plants to superinfection with *Agrobacterium* strains carrying wild-type octopine or nopaline Ti plasmids. This effect may be important for the genetic engineering of plants which already contain a pGV3850-related T-DNA with a gene of interest within the pBR portion of pGV3850 (see below); these plants could then be superinfected with an *Agrobacterium* containing pGV3850 plus another different gene of interest.

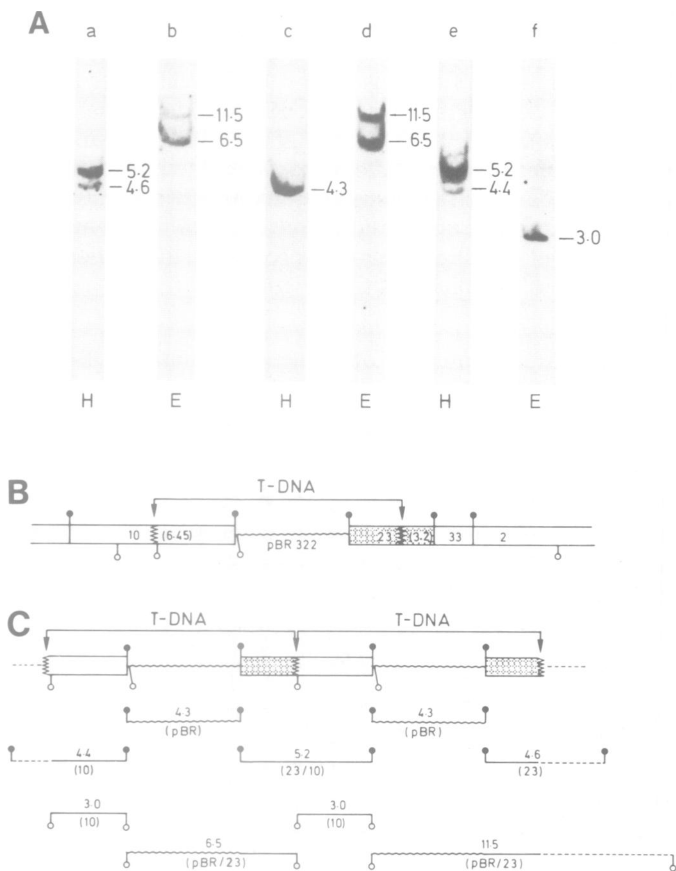

Fig. 4. Analysis of the T-DNA structure in pGV3850-transformed tobacco plants. Total DNA was prepared from the nopaline-positive pGV3850-transformed plant shown in Figure 3B. The purified DNA was digested with either restriction endonuclease *Hind*III or *Eco*RI and separated on a 1% agarose gel. The gel was blotted onto nitrocellulose filters and hybridized to radioactive probes homologous to purified restriction fragment *Hind*III-23 of pTiC58 **(A)** (**a** and **b**); to pBR322 (**c** and **d**); or to purified restriction fragment *Hind*III-10 (**e** and **f**). The numbers refer to the sizes of the radioactive DNA bands in kilobase pairs (kb), and H and E refer to restriction endonucleases *Hind*III and *Eco*RI, respectively. The faint upper bands visible in **e** and **f**, which are not labeled in the figure, are not reproducible and likely represent partial digestion products which occurred in the reactions shown in **e** and **f**. **(B)** The T-DNA region of pGV3850. The vertical bars and the numbers refer to *Hind*III restriction endonuclease fragments of pTiC58. The sizes of *Hind*III fragments 10 and 23 are given in kb in brackets. Fragment 10 is shown as a white box, fragment 23 as a stippled box, and the borders of the T-DNA as jagged lines. Restriction endonuclease sites for *Hind*III (●) and *Eco*RI (○) are indicated. **(C)** Results of the Southern blot hybridization analyses shown in **A**. We show two T-DNA copies arranged in a tandem structure since one of the bands (5.2 kb) hybridizes with probes homologous to both *Hind*III fragments 10 and 23. The numbers refer to the sizes of the fragments in kb which are also found in **A**. The indications below each fragment in brackets refer to probe(s) which give positive hybridization. All the symbols are as in **B** except that the dashed line indicates plant DNA sequences.

Analysis of the structure of the T-DNA in tobacco cells transformed by pGV3850

As a further check that these nopaline-positive tissues were indeed transformed with the minimal T-DNA of pGV3850, we analyzed total DNA prepared from the pGV3850-transformed plant shown in Figure 3B by restriction endonuclease and Southern blot hybridization analyses. The DNA was digested with either *Hind*III or *Eco*RI, and hybridized with probes for either the left border (pTiC58 *Hind*III fragment 10 probe), right border (pTiC58 *Hind*III fragment 23 probe), or middle (pBR322 probe) of the T-DNA of pGV3850. We were especially concerned that the pBR322 sequences were intact (see below, application of pGV3850), and this was found to be true. In addition, with the left and right probes we obtained border fragments as expected and neither Ti plasmid fragments *Hind*III-23 or *Hind*III-10 were observed.

Figure 4A shows the Southern blot hybridization patterns; Figure 4B shows the T-DNA of Ti plasmid pGV3850 prior to transfer to plant cells; and Figure 4C summarizes and presents a diagram of the T-DNA structure in transformed plant cells derived from the hybridization results of Figure 4A. The results suggest that the T-DNA is arranged in tandem copies; for simplicity in the drawing we show only two copies, although we estimate there are between two and five copies of pGV3850 T-DNA in the transformed plant cell DNA analyzed here. There are several reasons for proposing a tandem structure; (i) the intensity of hybridization of the internal pBR322 band when transformed plant cell DNA is digested with *Hind*III is greater than any of the border fragment hybridizations; (ii) there is a *Hind*III border fragment (5.2 kb) which hybridizes with probes homologous to *Hind*III fragment 23 and *Hind*III fragment 10 of the Ti plasmid; the intensity of hybridization is also greater as would be expected for a fragment present in several copies; (iii) we find only one right border fragment (*Eco*RI fragment of 12 kb or *Hind*III fragment of 4.6 kb) and one left border fragment (*Hind*III fragment of 4.4 kb); (iv) digestion with *Eco*RI does not reveal a left border fragment as the *Eco*RI site lies within 50 bp of this border (Yadav *et al.* 1980,1982; Zambryski *et al.*, 1982); however, we also obtain the expected internal *Eco*RI fragment of 3 kb. This latter internal fragment also hybridizes with a higher intensity as would be expected for multiple copies of the T-DNA.

These results represent the first analysis of plant cell DNA transformed with a Ti plasmid lacking all the internal portion of the T-DNA and demonstrate that the T-DNA borders are sufficient to allow transfer and stable integration of the DNA contained between these borders.

The use of pGV3850 as a Ti plasmid vector for the introduction of foreign DNA into plant cells

A useful application of pGV3850 is that the pBR322 sequences which are contained between the T-DNA borders can be used directly as an acceptor site for the insertion of foreign DNA. A single cross-over event in *Agrobacterium* will introduce a cloning vector containing any foreign gene of interest which is inserted in pBR322 (or its derivatives) into the modified T-DNA of pGV3850 (Figure 5). It may be of practical advantage that the DNA to be introduced is contained in

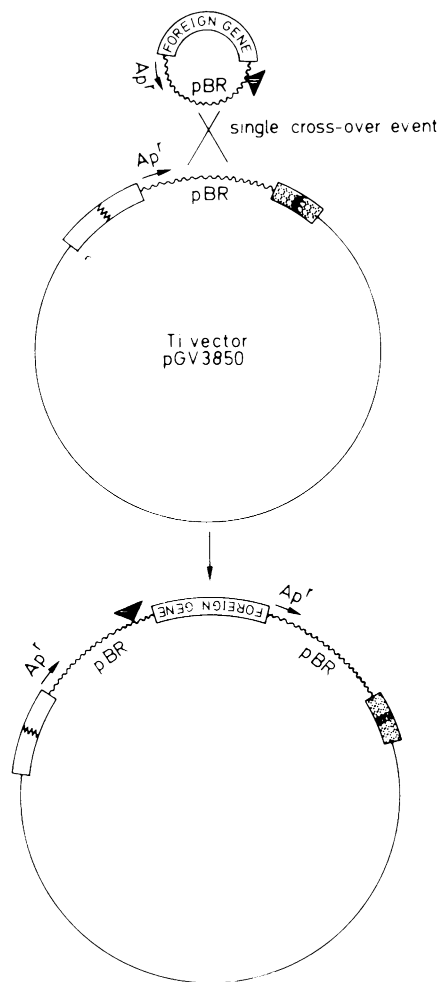

Fig. 5. Usefulness of Ti plasmid vector pGV3850 as a recipient for any foreign gene of interest whose expression is to be monitored in whole plants. A DNA of interest (foreign gene) can be cloned by recombinant DNA techniques into a pBR-type plasmid vehicle in *E. coli*. This recombinant plasmid can be mobilized to *Agrobacterium* using helper plasmids (Van Haute *et al.*, 1983). A single recombination event will result in the co-integration of the recombinant plasmid containing the gene of interest with pGV3850. By using selectable antibiotic resistance marker genes linked to the foreign gene, the co-integrate structure is stably maintained in *Agrobacterium* despite the duplication of the pBR sequences. As shown, the alignment of homologous pBR regions for recombination results in the reversal of the orientation of the foreign gene. The symbols are as in Figures 1 and 4, except the triangle with the diagonal lines indicates a second antibiotic resistance marker gene other than ampicillin resistance gene of pBR322.

a vector with an additional resistance marker gene to the Ap^R already present in pGV3850. Such a marker can be used as a genetic selection for the transfer of the intermediate cloning vector from *Escherichia coli* to *Agrobacterium*. This resistance marker can be contained either within the vector sequences such as chloramphenicol resistance (Cm^R) for pBR325 (Bolivar, 1978) or kanamycin resistance (Km^R) for pKC7 (Rao and Rogers, 1979), or within the foreign DNA which is to be tested in the plant cell. The resulting Ti plasmid contains a duplication of pBR322 sequences. One might expect this to lead to instability of the inserted DNA. However, this is not a problem in *Agrobacterium* because the gene of interest is linked to an antibiotic resistance marker that can be maintained by growth on selective medium. An instability of the sequences in the resulting transformed plant DNA is not expected as repeated DNA sequences are a normal part of the plant genome, and the T-DNA itself is often (also, as shown, in the pGV3850-transformed tissue investigated here) organized as an array of tandemly repeated copies in transformed plant cells (Lemmers *et al.*, 1980; Zambryski *et al.*, 1980, 1982).

An experiment was designed to test the stability of co-integrate derivatives of pGV3850. The *Eco*RI fragment 7 of the T-DNA of the octopine Ti plasmid (De Vos *et al.*, 1981) was cloned in a plasmid vector containing resistance marker genes (ampicillin, tetracycline, streptomycin, spectinomycin and sulfonamide) as well as pBR322 sequences (J. Leemans and R. Deblaere, unpublished results). This *Eco*RI fragment carries several of the oncogenic genes encoding a tumorous phenotype including hormone-independent growth (Garfinkel *et al.*, 1981; Leemans *et al.*, 1982; Willmitzer *et al.*, 1982). The co-integrate between pGV3850 and the intermediate plasmid vector (Leemans *et al.*, 1981) carrying this *Eco*RI fragment in *Agrobacterium* was used to infect tobacco plantlets. The resulting tissue is transformed and has been growing as a tumor on hormone-free medium for 10 months so far without any alteration of its properties; in addition, the tissue remains nopaline-positive (Figure 2l).

Discussion

In the past, the insertion of foreign DNA into the T-DNA region of the Ti plasmid required a further cloning of the DNA of interest into a plasmid vector which also contained a portion of the T-DNA region to serve as a region of homologous recombination with the acceptor Ti plasmid (Leemans *et al.*, 1981; Matzke and Chilton, 1981). The development of techniques for direct mobilization of plasmids containing pBR322 sequences to *Agrobacterium* (Van Haute *et al.*, 1983) makes pGV3850 an extremely versatile acceptor Ti plasmid. Assuming a transmission frequency from *E. coli* to *Agrobacterium* of 4.5×10^{-3} (Van Haute *et al.*, 1983), we have observed a recombination frequency of 2×10^{-2} for the homology region of the pBR322 sequence of pGV3850 (L. Herrera-Estrella, D. Inzé and P. Zambryski, unpublished results). Besides insertion of defined clones containing a pBR322 sequence by a single cross-over event into the acceptor Ti plasmid pGV3850, this Ti plasmid can also be used as a recipient for cloned banks of DNA in pBR322 or its derivatives in a 'shotgun cloning' experiment. The total population of hybrid plasmids in *Agrobacterium* can be used to infect plant cells and can be subsequently screened for expression of the gene(s) of interest.

Recently, foreign genes have been successfully expressed in plant cells using a vector which contains the promoter sequences of the Ti plasmid-specific nopaline synthase gene (Herrera-Estrella *et al.*, 1983a,1983b). Four different coding sequences were inserted behind the *nos* promoter and were shown to be properly expressed following infection of plant cells with wild-type Ti plasmid carrying these chimeric genes. The four different coding sequences used were octopine synthase, and three others for bacterial antibiotic resistance genes: the aminoglycoside phosphotransferase (APH(3′)II) conferring kanamycin resistance from Tn*5* (Davies and Smith, 1978), the methotrexate-insensitive dihydrofolate reductase (DHFR Mtx^R) conferring methotrexate resistance from R67 (Fling and Elwell, 1980; O'Hare *et al.*, 1981), and chloramphenicol acetyl transferase conferring chloramphenicol resistance from pBR325 (Bolivar, 1978). Furthermore, the expression of the antibiotic resistance genes could be used as a specific selection for transformed tobacco protoplasts grow-

ing as a mixed population in tissue culture (Herrera-Estrella *et al.*, 1983b).

As a further test of the vector properties of pGV3850 as well as of the expressibility of foreign genes in plants, we have recombined these various chimeric constructions, which contain the *nos* promoter linked to antibiotic resistance genes, into pGV3850. The co-integrates have been used to infect tobacco protoplasts and resistant calli have been isolated (M. De Block, L. Herrera-Estrella and P. Zambryski, unpublished results); these calli will be grown further to check for the physical presence of the foreign genes. These tissues can be subsequently regenerated into complete plants to test for the expression of the resistance genes in differentiated tissues. The use of selectable marker genes in combination with non-oncogenic Ti plasmids, such as pGV3850, makes it possible to select for transformed plant cell protoplasts in tissue culture and subsequently regenerate whole plants containing the inserted DNA. For the future, one has to simply link other genes of interest to the selectable marker gene prior to transfer to the acceptor Ti plasmid.

A simple approach to obtain transformed plants by inoculation of decapitated tobacco plantlets with *Agrobacterium* containing pGV3850 is presented. As there is no tumor response, the wound surface is removed and cultivated in tissue culture as a callus. A subsequent transfer of this wound callus to regeneration medium results in the production of transformed plantlets which can be easily distinguished from untransformed plantlets by a nopaline test. Furthermore, the frequency of obtaining transformed shoots is rather high. These results demonstrate that it is not necessary to have a protoplast cell culture system in order to have efficient transformation; it is likely that the method utilized here can be applied to the inoculation of transformed plants from species which are not amenable to protoplast cultivation.

The vector pGV3850 makes use of the natural transfer properties of the Ti plasmid; only those genes which interfere with normal plant differentiation have been removed. Thus, the most important aspect of pGV3850-transformed cells is their capacity to regenerate into complete plants. These plants can be derived from single cells and the regeneration process itself is extremely simple, requiring only minor changes in tissue culture conditions. These results clearly open up several areas for investigation of plant biology. For example, we may now ask questions about tissue-specific regulation of genes. Genes isolated from one cell type can be re-added to plant cells via pGV3850 to investigate whether or not they are expressed again in the appropriate cell; these results should lead to the identification of tissue-specific controlling signals. In addition, we can monitor the effect(s) of completely foreign genes whose properties we may wish to transfer into plants. Basic plant processes, such as chloroplast function or hormone physiology may also be studied. Perhaps the most interesting subject will be the study of genes which are developmentally regulated. Thus, evidence has been presented that the Ti vector has evolved to a point where it is ready to be used to genetically engineer whole plants; it remains for us to turn our efforts toward the isolation of particular genes whose expression we wish to study.

Materials and methods

Conditions of bacterial growth

The *E. coli* and *Agrobacterium* strains to be analyzed were cultured as described by Joos *et al.* (1983).

Bacterial conjugation

Conjugations involving *E. coli* and *Agrobacterium* strains were performed as described by Van Haute *et al.* (1983).

DNA preparation

Total *Agrobacterium* DNA was prepared using techniques described by Dhaese *et al.* (1979).

Total plant DNA was prepared in the manner previously described (Lemmers *et al.*, 1980), except that wet tissue was used directly and the extraction buffer was two times concentrated. The tissue was rapidly frozen in liquid nitrogen and homogenized in a Waring Blender. One volume (equivalent to the wet weight of the plant tissue) of 2 x extraction buffer was added and the DNA was isolated as before (Lemmers *et al.*, 1980).

Restriction digests of DNA

All restriction enzyme digests were incubated in the TA buffer described by O'Farrell *et al.* (1980).

Hybridization conditions

Hybridization and washing of nitrocellulose filters was performed at 68°C in 3 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M Na-citrate) and 0.2% SDS.

Oncogenicity tests

The different *Agrobacterium* strains have been infected on four different test plants in order to test their oncogenic capacities. Tobacco (*Nicotiana tabacum* cv. Wisconsin 38) and *Petunia hybrida* (var. Mitchell) plantlets, and potato (*Solanum tuberosum* var. Bintje) and carrot (*Daucus carota*) slices have been infected as described previously (Leemans *et al.*, 1982; Joos *et al.*, 1983).

In vitro cultivation of plant tissues

Hormone-independent crown gall tumor tissue was grown *in vitro* on plates containing LS medium (Linsmaier and Skoog, 1965) with 1% sucrose and 0.8% agar. Hormone-dependent tissue induced by pGV3850 was grown continuously on the same medium containing 1 mg/l NAA and 0.2 mg/l BAP. Shoots were regenerated from callus tissue by placing it on LS medium containing 1 mg/l BAP. Tissues were made free of *Agrobacterium* by growing primary wound tissue as well as the first few subcultures of this tissue on medium which also contained 500 μg/ml cefotaxime (Claforan, Hoechst).

Nopaline detection in plant tissues

A small amount (50 mg) of either callus tissue or leaf material was crushed with a glass rod in an Eppendorf centrifuge tube. After centrifugation for 5 min, a part of the supernatant (2 μl) was spotted directly onto Whatman paper and the extracts were electrophoresed and stained for the presence of opines as described (Aerts *et al.*, 1979).

Acknowledgements

We would like to thank Luis Herrera-Estrella, Henri De Greve, and Jean-Pierre Hernalsteens for their critical advice during the course of this work. In addition, we thank Ms. Martine De Cock, Karel Spruyt and Albert Verstraete for their help in preparing this manuscript. This research was supported by grants from the 'Kankerfonds van de Algemene Spaar- en Lijfrentekas', from the 'Instituut tot Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw' (I.W.O.N.L. 3849A), from the Services of the Prime Minister (OOA/12052179), and from the 'Fonds voor Geneeskundig Wetenschappelijk Onderzoek' (F.G.W.O. 3.001.82) to J.S. and M.V.M. H.J. and J.L. were supported by fellowships of the I.W.O.N.L., and P.Z. is indebted to the USDA (grant #5901-0410-9-0374-0) for support and to the EMBO for a long-term fellowship.

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