Introduction of Genetic Material into Plant Cells

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Summary. The tumor-inducing (Ti) plasmid of the soil microorganism *Agrobacterium tumefaciens* is the agent of crown gall disease in dicotyledonous plants. The Ti plasmid contains two regions that are essential for the production of transformed cells. One of these regions, termed transfer DNA, induces tumor formation and is found in all established plant tumor lines; the other, termed the virulence region, is essential for the formation but not the maintenance of tumors. Transfer DNA, which transfers to the plant genomes in a somewhat predictable manner, can be increased in size by the insertion of foreign DNA without its transferring ability being affected. The tumor-causing genes can be removed so that they no longer interfere with normal plant growth and differentiation. This modified Ti plasmid can thus be used as a vector for the transfer of foreign genes into plants.

Many techniques have been proposed for the transfer of DNA to plants such as direct DNA uptake, microinjection of pure DNA, and the use of viral vectors. To date, the simplest and most successful method has made use of the natural gene vector system of *Agrobacterium tumefaciens*.

Agrobacterium tumefaciens is a soil microorganism that is capable of infecting a broad assortment of dicotyledonous plants after they have been wounded (*1*). As a result of this infection, the wound tissue begins to proliferate as a neoplastic growth commonly referred to as a crown gall tumor. Once induced, the tumors no longer require the presence of bacteria to continue growing (*2*). Among the most important of the new properties of these transformed cells are first, that they can grow axenically in vitro without the hormone supplements normally required by plant cell cultures, and second, that they can synthesize a variety of compounds unique to tumors. The latter compounds, which are termed opines, can be metabolized specifically by the bacteria responsible for inciting the tumor (*3*).

The genes responsible for hormone-independent growth, for the ability to induce opine biosynthesis, and for the ability to metabolize opines are all encoded by the tumor-inducing (Ti) plasmid of *A. tumefaciens* (*4–6*). A specific portion of the Ti plasmid, the T region or T-DNA, is transferred from the plasmid to the nucleus of a susceptible plant host (*7*). There the DNA is integrated into plant chromosomes as a unit, with discrete end points (*8, 9*), which contains the genes responsible for opine biosynthesis and for tumor growth (*5, 6, 10–12*).

We discuss here some of the recent experiments that indicate (i) how DNA might be transferred from the bacterium to the plant, (ii) which sequences are involved in the integration of plasmid DNA into the plant chromosome, and (iii) how the integrated DNA appears to influence the growth properties of the infected cell. On the basis of data from these experiments it has been possible to transform the Ti plasmid into a simple and reliable gene-transfer vector and to use this vector to investigate tumorigenesis and to introduce prototypes of genes that may be used to investigate the genetic control of plant development. These techniques may form the basis for new developments in research on agriculturally important plant species.

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Ti Plasmid Sequences Essential for Tumor Formation

Agrobacteria that have been cured of their Ti plasmid no longer induce tumors (*13*); thus the first attempts to determine the identity and location of the genes responsible for tumorigenesis concentrated on the Ti plasmid. By means of transposon mutagenesis (*5, 6, 10*) and, more recently, by analysis of the effects of substantial deletion mutations (*14, 15*), it has been possible to demonstrate that the Ti plasmid contains two distinct and separate regions that are essential to produce transformed cells (Fig. 1).

The first region, the T-DNA, contains all of the Ti plasmid sequences found in most established tumor lines. In some plasmids, for example pTiA6NC, pTiAch5, and pTiB6S3, this region is divided into two adjacent independently acting DNA segments (*9*), one of 13.6 kilobases (left T-DNA) and one of approximately 7 kb (right T-DNA). A characteristic of this type of plasmid is that one of the genes of the left T-DNA encodes the synthase for one of the opines, octopine (*16*). Other plasmids, for example, pTiT37 or pTiC58, transfer a single T-DNA of 23 kb (*8*) which carries the gene encoding the synthase for nopaline, another opine (*5, 17*). Both T-DNA's have been studied extensively to identify the functions responsible for tumor formation and for transfer to plants.

The T-DNA's from both classes of Ti plasmids encode a variety of polyadenylated transcripts (*18*), six of which map in a common 9-kb DNA segment (*19*). These six transcripts are arranged in the order 5, 2, 1, 4, 6a, 6b across the T-DNA region (Fig. 2). Throughout this article, T-DNA genes will be referred to by the number given to their transcript, for example, gene *1* encodes transcript 1. Mutations in the nonhomologous regions of the two types of T-DNA, and in the genes for transcripts 5, 6a, and 6b, have either no or limited influence on tumor formation (*11, 12, 20*).

Three of the genes of the common DNA appear to be directly responsible for tumor formation. Normal crown gall tumors are unorganized, whereas tumors obtained from mutants containing insertions in gene *4* (the Roi locus) allow root formation on most of the plants on which they are tested (*11, 20, 21*). Tumors induced by mutants of genes *1* or *2* (the Shi locus) grow as green calli that sprout both normal and malformed shoots (*11, 12, 20, 21*). In analogy to what is known about plant growth regulators, the effect of gene *4* can be thought of as "cytokinin-like," so that inactivating it might result in a low cytokinin to auxin ratio, and hence, to root formation. Similarly, the combined effects of genes *1* and *2* can be thought of as "auxin-like," since mutations in either gene appear to increase

the cytokinin to auxin ratio of tumors, and this might in turn lead to shoot formation (*21, 22*). Therefore, the undifferentiated appearance of a crown gall tumor and its ability to grow without exogenous hormones must reflect the combined activities of the products of genes *1*, *2*, and *4*. Some support for this model comes from studies on growth of Shi$^-$ or Roi$^-$ tumors (Fig. 2) in vitro. It has been found that tobacco tumors induced by T-DNA mutants in gene *4* require cytokinin in vitro for optimal growth (*20, 23*). Similarly, tobacco tumors induced by T-DNA mutants in the Shi locus are auxin-dependent in tissue culture unless they can form sufficient shootlike structures to manufacture the necessary auxin (*24*).

The second portion of the Ti plasmid containing sequences essential for tumor formation has been termed the virulence or *vir* region (Fig. 1) in order to distinguish the genes encoded there from the oncogenes of the T-DNA. The DNA sequences of the *vir* region have not been found in established tumor lines, and therefore are not essential for tumor maintenance (*8, 9, 25*).

These functions were studied in greater detail by transposon insertion mutagenesis of the *vir* region of an octopine Ti plasmid (*26, 27*). Each avirulent mutation in the *vir*-region was tested for its ability to be complemented *in trans* in the bacterium by overlapping cosmid clones carrying other *vir* insertion mutations. These results demonstrated that the *vir* functions are organized into at least ten independent complementation groups.

It is not clear how the *vir* functions contribute to the formation of crown gall tumors. A number of mutations have been identified that markedly reduce the specific affinity of *Agrobacterium* cells for plant cells, but all of these map to the bacterial chromosome (*28*), and it is possible that the Ti plasmid does not play a major role in the formation of these sorts of contacts (*28, 29*). Furthermore, *vir* mutants cannot complement each other when coinfected on the same wound (*27*). This indicates that none of the products of the *vir* functions is readily diffusible between bacteria.

Recent experiments have indicated that the T and *vir* regions act as physically self-contained units. For these studies, the genes of each region were cloned on independent replicons. It was found that neither region was oncogenic by itself, but that the two together complemented each other to stimulate tumor formation (*30*). Moreover, plasmids con-

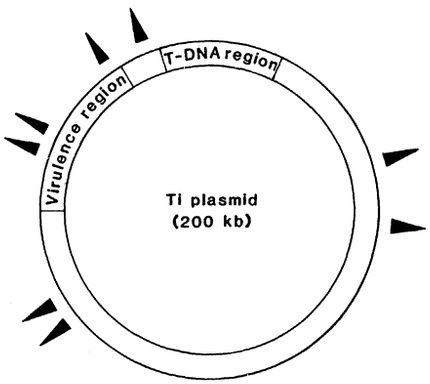

Fig. 1. Schematic diagram of the Ti plasmid. The relative locations of the two portions of the nopaline Ti plasmid that govern tumor formation are indicated as the virulence and T-DNA regions. The triangles surrounding the plasmid show the approximate locations of oncogenes that have been inserted in a plasmid that lacks the oncogenes of the normal T-DNA region (*38*).

taining the T region of a nopaline plasmid (pTiT37 or pTiC58) complements the *vir* region of an octopine plasmid (pTiAch5 or pTiB6S3) and vice versa, demonstrating that neither set of genes contains oncogenic functions specific to one class of plasmid (*14*).

Transfer of the T-DNA from *Agrobacterium* to Plant Cells

The interaction between *Agrobacterium* and plant cells sets in motion a chain of events that ultimately transfers the T-DNA from the Ti plasmid into the plant nuclear DNA. To discuss how this transfer occurs, one must first point out which portions of the Ti plasmid are associated with the integrated DNA and which portions may be essential for transfer to the plant.

There is little variation in the ends of the T-DNA from different tumor cell lines analyzed to date. In all cases, the homology between sequences present in the Ti plasmid and those in the tumor DNA ends within, or proximal to, a 25-base pair "terminal sequence" (Fig. 3) that flanks the T region of the plasmid as direct (albeit, imperfect) repeats (*31–34*). The T-DNA borders occur one base before or at the first base pair of the right copy of this sequence in at least three different tumor lines (*31, 34*). The left border of the integrated T-DNA seems to be more variable than the right, but nonetheless occurs within 100 bp of the left 25-bp sequence (*31, 33*).

Extensive deletions have been made at each of the ends of the T region in order to determine whether each end is functionally equivalent to the other. A deletion of the right end makes the T region virtually avirulent on most plant species (*5, 20, 35*). By contrast, deletions of the left end have no apparent effect on the tumor-forming ability of the T region (*20*). In fact, recent experiments show that a clone containing only the nopaline synthase (*nos*) gene and the right border is fully capable of transferring the *nos* gene to the plant when it is inserted in the *vir* region of a Ti plasmid or when it is part of a free replicon that is complemented by a Ti plasmid in *Agrobacterium* (*36*). Since a "terminal sequence" is closely associated with normal T-DNA ends and also with the end of an unusually short T-DNA (*37*), it is thought important for T-DNA integration. It is likely that there are related sequences elsewhere on the plasmid that can substitute for the end that is missing in these mutated T regions.

The observations on the differences between the ends of the T region can be interpreted in at least two ways. One is to assume that the requirements for integration are more stringent for the right end of the T-DNA than for the left. The other is to propose that the requirements for integration are similar but that the right end contains another function, such as a transit sequence that directs the transfer of adjacent DNA to plants.

There are also two hypotheses concerning the actual process of T-DNA transfer. One possibility is that the T region separates at the borders from the remainder of the plasmid during the normal course of infection and enters the plant cell alone. Alternatively, the whole Ti plasmid might enter the plant cell and be lost later, after the T-DNA has entered a host chromosome. The latter proposition has been tested by use of a unique set of plasmids (*38*). These are derived from a nononcogenic mutant (pGV3850) of the plasmid pTiC58 which lacks the *onc* genes present in the central core of the T-DNA (Fig. 4). This plasmid still contains both ends of the T-DNA region required for transfer and integration of DNA into plants as well as the gene for the synthesis of the opine nopaline (*39*). Next, a clone containing the oncogenes but lacking the left and right ends of the T-DNA region was inserted into eight different sites (Fig. 1) around the map of this nononcogenic Ti plasmid. Five of the eight aberrantly reconstituted plasmids (those that do not have insertions that inactivate *vir* genes) induce tumors, although not as efficiently as normal plasmids (*38*). In contrast, when the oncogenes and the nononcogenic Ti

plasmid are maintained as separate replicons, no tumors can be produced.

The interpretation of these observations is that the genes of the central core of the T-region can induce tumors but cannot promote their own transfer or stabilization in plant cells. Consequently, these genes enter the plant only when linked to a plasmid containing transfer or integration sequences, or both. The new location of the oncogenes appears to be unimportant, suggesting that most, if not all, of the Ti plasmid might be able to enter cells of the plant host. It is possible, however, that transfer of sequences outside of the normal T region borders represents errors in discrimination by the transfer apparatus. Such rare events would be amplified by the very nature of tumorous growth to the point where they can be seen. These results are notable, but further tests are warranted to determine whether the whole of the Ti plasmid enters the plant cell during the course of normal infection.

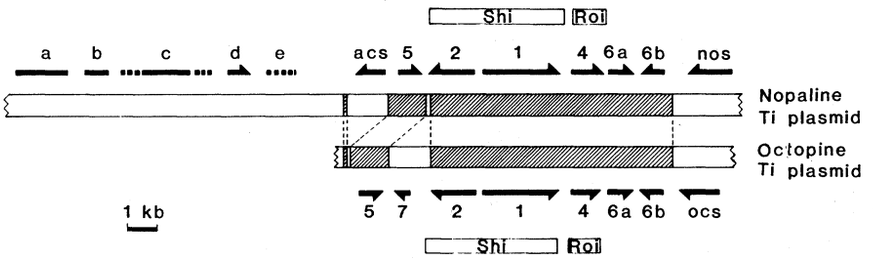

Fig. 2. The genetic organization of the T-DNA's of nopaline and octopine Ti plasmids. The nomenclature and locations of the polyadenylated transcripts are shown as either bars or arrows according to whether the direction of transcription is known (*18*). The upper T-DNA is characteristic of the pTiC58 T-DNA which contains the genes for nopaline synthase (*nos*) and the synthase for an opine, agrocinopine (*acs*). The lower T-DNA is characteristic of pTiB6S3 which encodes octopine synthase or *ocs*. The hatched areas mark the regions of homology (common DNA) between the two T-DNA's (*19*). The box with the abbreviation *Shi* indicates which transcripts control shoot inhibition; *Roi* indicates the transcript that controls root inhibition. The ends of the T-DNA that are used in integration into plant genomes are presented as jagged lines.

Design of a Vector for Foreign DNA Transfer

Although the exact role of the T region terminal sequences requires further study, there is no doubt that the T-DNA can transfer to the plant genome in a predictable and rather precise manner. Furthermore, it has been possible to increase the size of the T-DNA by at least 14 kb through the insertion of foreign DNA without affecting transfer (*40*), and it is likely that even larger DNA inserts can be transferred equally well. Current research is focused on the design of modified Ti plasmid derivatives that will be useful for genetic engineering of plants. There are two major characteristics that need to be incorporated into these new Ti vectors: (i) they must allow efficient DNA transfer to plant cells without interfering with normal plant growth and development, and (ii) they must allow foreign DNA to be inserted easily in between the terminal sequences flanking the T-region.

Recently, such a Ti plasmid vector was constructed (*39*). pGV3850 is a nononcogenic derivative of the nopaline Ti plasmid C58. This vector utilizes the natural transfer properties of the Ti plasmids and has the following characteristics: (i) the T-DNA border regions and all the contiguous Ti plasmid sequences outside the T-DNA-region; (ii) the DNA near the right T-DNA border encoding nopaline synthase as a marker for transformed cells; and (iii) the internal T-DNA genes that determine the undifferentiated crown gall phenotype have been deleted and are replaced by the commonly used cloning vehicle, pBR322. The pBR322 sequence contained between the T-DNA border regions serves as a region of homology for recombination to introduce foreign DNA's cloned in pBR322 derivatives. The structure of pGV3850 as well as its use as an acceptor plasmid are shown in Fig. 4.

The use of this system involves straightforward genetic techniques: a single cross-over event between the pBR322 region of pGV3850 and the pBR region of the plasmid carrying the gene of interest produces a cointegrated plasmid that can be used to transform plants. The direct mobilization of plasmids containing pBR sequences from *Escherichia coli* to *Agrobacterium* is possible because of the recent progress in the use of helper plasmids able to mobilize and transfer pBR (*41*). As pBR itself cannot replicate in *Agrobacterium*, the only way in which it can be maintained is by recombination with the homologous region in the Ti plasmid. As shown in Fig. 4, the cointegrated structure contains a duplication of the pBR sequences; however, this apparently does not lead to instability. The cointegrate can be maintained in *Agrobacterium* by including and selecting for a drug resistance marker other than ampicillin in the pBR vehicle containing the foreign gene. It is unlikely that the cointegrate will be unstable in the plant cell as the plant genome is composed of much repeated DNA, including, at times, the T-DNA itself which can be present in tandem copies (*8, 34*).

We have studied the transformation of plant cells with pGV3850 and have demonstrated that the shortened T-DNA can be efficiently transferred to several plant species including tobacco, petunia, carrot, and potato. Furthermore, in vitro cocultivation of tobacco protoplasts with *Agrobacterium* containing either wild-type Ti plasmid or pGV3850 yields transformed cells at the same frequency (*42*). Using infected tobacco as a model system, we were able to regenerate plants containing the T-DNA of pGV3850 and to confirm the structure of this T-DNA by Southern hybridization. These results are the first demonstration that the T-DNA borders alone are sufficient to allow transfer and stably integrate the DNA contained between the borders.

The presence of the nopaline synthase (*nos*) gene in this vector makes it easy to monitor the efficiency of transformation using pGV3850. For example, several independent tobacco plantlets were inoculated with pGV3850. The tissue at the site of inoculation was removed, propagated as callus in tissue culture, and then tested for the presence of nopaline; 25 percent of the calli were *nos* positive. These calli, which contained a mixture of transformed and untransformed cells, were then transferred to a medium that induces plant regeneration. Between 9 and 78 percent of the regenerated plants derived from different *nos*-positive calli contained nopaline, indicating that transformation was remarkably efficient.

Use of pGV3850 to Study the Genes Involved in Tumorigenesis

The following experiments demonstrate the usefulness of the plasmid vector pGV3850 and prove that genes can be transferred and stably maintained in the plant cell genome by means of this system. The genes chosen were those of the

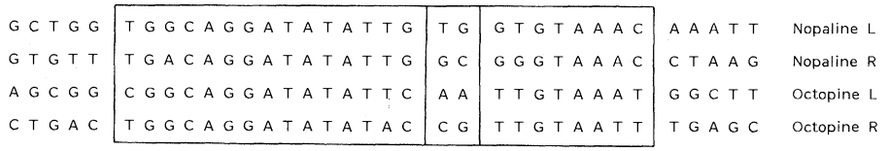

Fig. 3. Comparison of the 25-bp terminal sequence at the T-DNA borders of the nopaline and octopine Ti plasmids. The box indicates the homology between the terminal sequences flanking the (*L*, left; *R*, right) T-DNA regions of nopaline pTiC58 (or pTiT37) and octopine pTiB6S3 Ti plasmids (*31–34*). The two bases at positions 16 and 17 that are not conserved among the four sequences are also enclosed.

T-DNA region itself that encode functions that induce tumors. In the first experiment the entire region of the T-DNA that is responsible for the wild-type tumor phenotype was cloned in a pBR-derived plasmid, and recombined into the pBR322-region of pGV3850 (*39*). Agrobacteria containing this cointegrate have been used to infect plants and have produced wild-type tumors. This tissue is stable in vitro, contains nopaline synthase activity, and has been growing as a tumor for over 10 months.

In a second series of experiments, pBR-derived clones of each of the individual T-DNA oncogenes have been recombined with pGV3850 (*43*). These experiments were designed to analyze the contribution of each *onc* gene to the development of a tumor and have fulfilled two purposes. First, they have demonstrated that the T-DNA genes *1*, *2*, and *4* can be expressed independently of all other genes, and second, that only gene *4* can induce tumors by itself. In order to do this, specific T-DNA fragments in a pBR vehicle were introduced into the T region of pGV3850. Strains carrying each construction were coinfected on the same wound with a strain carrying a T-region insertion mutation. These experiments demonstrated that Ti plasmids that could transfer only gene *1* to plants could complement the Shi^- phenotype of a strain with a mutation in gene *1*. Similarly, plasmids that could transfer only gene *2* could complement a strain with a mutation in gene *2*. Neither gene *1* nor gene *2* could induce tumors by themselves, but coinfections of a strain carrying only gene *1* with another strain carrying only gene *2* produce small tumors on tobacco. Strains carrying gene *4* produced tumors without the aid of other T-DNA genes; in vitro these tumors produce shoots.

These experiments together with those discussed earlier indicate that there are two separate pathways of tumorigenesis: one is a shoot-suppressing and root-stimulating (auxin-like) pathway encoded by genes *1* and *2*, and the other is a root-suppressing, shoot-stimulating (cytokinin-like) pathway encoded by gene *4*. As techniques are developed to modulate the levels of expression of these genes, for example, by putting them under the control of inducible promoters, it is hoped that further insight about the mechanism of action of T-DNA genes can be gained.

Development of Systems to Express Genes in Plants

One of the major goals in the use of *Agrobacterium* is to take advantage of its natural properties in order to introduce and express new genes in plants. The first successful attempts to introduce foreign DNA into plant cells were done with bacterial transposons. Tn*7* (*40*) and Tn*5* (*11*) were inserted in vivo into the T-DNA of Ti plasmids pTiT37 and pTiA6NC, respectively, and were found to be efficiently cotransferred with the T-DNA. The genes encoded by these transposons failed to be expressed, presumably because the eukaryotic transcriptional machinery of the plant did not recognize the promoter sequences of these prokaryotic genes. Further attempts to express heterologous eukaryotic genes, such as the yeast alcohol dehydrogenase gene (*23*) or genes from mammalian cells, such as β-globin (*44*), interferon (*45*), and genes under control of the SV40 early promoter (*46*), showed that none of these genes was transcribed in plant cells. This suggests that specific transcription factors or signals that are required for their expression are present only in the cells or specific tissues of their original hosts.

It became obvious that the first step for the expression of heterologous genes in plants would require the use of transcriptional signals from a gene that is known to be functional in plants. So far, only a few sets of genes have been isolated, and most of these are highly regulated or specific for only a particular stage of the plant. For example, the leghemoglobin (*47*) or zein (*48*) and phaseolin (*49*) gene families are expressed only in either *Rhizobium*-induced nodules or in plant seeds, respectively. One of the best candidates to be used as a donor of transcriptional signals was the *nos* gene. It is known that this gene is normally expressed both in callus tissue and in most of the plant tissues regenerated from calli containing the opine gene (*50*).

The nucleotide sequence of *nos* (*51*) reveals that its controlling signals share most of the characteristics of other plant genes in particular (*52*), and of other eukaryotic genes in general (*53*). At the 5′ end, the *nos* gene contains sequences homologous to the TATA or Hogness box, 35 bp upstream of the start of transcription, and a sequence similar to the AGGA box consensus sequence for plant genes 60 to 80 bp upstream of the 5′ end of the transcript. At the 3′ end, it has the sequences AATAAA and AATAAT, approximately 135 and 50 bp from the polyadenylation site, which strongly resemble the consensus sequence similarly placed in animal genes (*53*). It is worth mentioning that most of the T-DNA genes seem to lack introns (*37, 51, 54*). This, however, is not exceptional since there are other plant genes that lack introns, such as the maize zein gene family (*48*).

A series of expression vectors has been constructed containing the promoter and terminator signals for transcription of the *nos* gene; in between these signals are unique restriction sites for the insertion of any desired coding sequence (*55, 56*). One of these vectors was first used to express a homologous gene, the T-DNA–encoded octopine synthase gene, and a heterologous gene, the bacterial chloramphenicol acetyltransferase gene from the plasmid vector pBR325 (*55, 57*). In both cases the *nos* promoter and termination signals are enough to produce a transcript that is recognized by the translation machinery of the plant cells to produce an active protein. This constitutes the first example of the expression of a foreign protein in plant cells.

As demonstrated for bacterial and mammalian cells, one of the first steps to the development of efficient transformation systems is the construction of dominant selectable markers. These allow cells that have acquired new genes via transformation to be selected and identified easily. For this reason, vectors based on the *nos* regulatory signal were tested for their ability to express bacterial genes that encode resistance to antibiotics, such as G418, kanamycin, or methotrexate, which are highly toxic to

plant cells. By means of these chimeric genes it was demonstrated that the proteins encoded by the aminoglycoside phosphotransferase II (APH II) from Tn*5* (*56, 58, 59*), aminoglycoside phosphotransferase I (APH I) from Tn*903* (*59*), or the dihydrofolate reductase that is methotrexate-insensitive from R67 (*56*) can be used to confer to plant cells resistance to kanamycin, G418, or methotrexate, respectively. In each case, the chimeric genes were transferred via the Ti plasmid to plant cells by an in vitro transformation technique. In this protocol, protoplasts that are undergoing cell wall regeneration are incubated with *A. tumefaciens* for 14 to 30 hours and then treated with antibiotics to kill the bacteria. After 1 to 2 weeks, small fast-growing plant cell colonies can be exposed to selective medium containing 50 to 100 μg of kanamycin per milliliter (*56, 58, 59*).

These experiments have shown that four different bacterial coding sequences are properly expressed and translated into active proteins in plant cells. Thus, the codon usage for the plant translation machinery may allow expression of any other bacterial, fungal, or mammalian gene, including those which could confer a useful trait to plant cells. At the same time, it is important to consider that there are not enough data to know whether all foreign proteins will be stable in plant cells once they are synthesized, especially if they are to be produced on a large scale.

These results demonstrate that the Ti plasmid can be used to transfer and express genes in plants. Now more sophisticated studies of gene regulation can be attempted. For example, one of the most exciting areas in plant biology is the study of how factors such as light regulate genes. Several members of the gene families involved with photosynthesis have been isolated, notably the families encoding the small subunit (ss) of the ribulose bisphosphate carboxylase (*60, 61*) and the chlorophyll-binding protein (*62*). The *ss* gene product participates in CO_2 fixation and the conversion of CO_2 into carbohydrates by using the energy obtained during photosynthesis with the aid of the chlorophyll-binding protein.

The expression of these two gene families has been shown to be light-induced at the transcriptional level in green tissue (*60, 61*). It is interesting to determine whether this type of light-regulated gene is controlled by the sequences 5′ to the promoter or by sequences in another part of this gene. Recently, a chimeric gene was constructed containing 900 bp of the promoter region of a small subunit gene isolated from pea (*61*), coupled to the CAT coding sequence from Tn*9* and the 3′ end sequence of the *nos* gene (*63*).

This chimeric gene was introduced into tobacco cells by using the Ti plasmid as a vector, and the light regulation of the CAT activity was assayed in green tissues. The 900 bp of the 5′ upstream region of the *ss* gene are sufficient to confer light-inducible expression of the CAT coding sequence (*63*). This finding constitutes the first evidence that the regulation of a plant gene is determined by sequences upstream of its promoter and, additionally, that a promoter from one plant species (pea) can function in the cells of another plant (tobacco).

One of the ultimate aims in the use of the Ti plasmids of *Agrobacterium* as a tool for studying gene expression in plants is to obtain fully differentiated plants that express sequences introduced by means of the Ti plasmid. This has recently been achieved (*64*) by inserting the NOS-APH(II) chimeric gene into the vector pGV3850 via homologous recombination through pBR322. Kanamycin-resistant colonies were obtained after in vitro transformation of tobacco protoplasts with *Agrobacterium* containing pGV3850::NOS-APH(II). The calli were grown in vitro until the tissue was axenic and were then transferred to medium containing a ratio of plant phytohormones that promotes plant regeneration. In contrast to calli induced with the wild-type Ti plasmids that are unable to differentiate in this medium, the kanamycin-resistant tissue transformed with pGV3850::NOS-APH(II) is able to produce shoots that later also form normal roots. In order to demonstrate that the

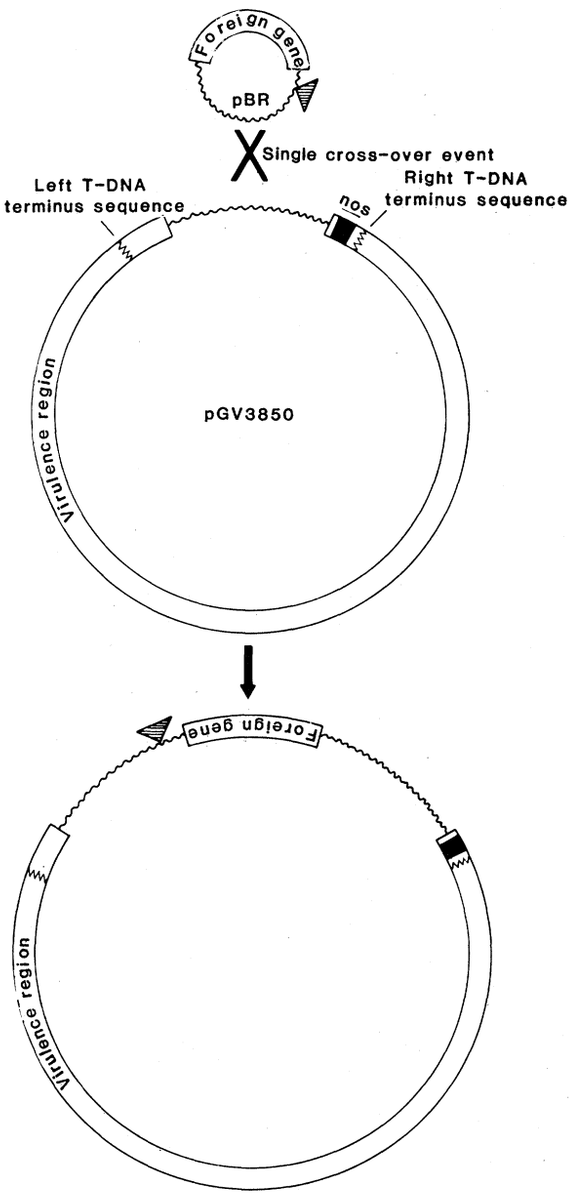

Fig. 4. Ti plasmid vector pGV3850, an acceptor for foreign genes whose expression is to be monitored in whole plants. The structure of Ti plasmid vector pGV3850 (*39*) is diagramed. It contains all Ti plasmid sequences (shown in white) except for the internal oncogenes of the T-DNA. The oncogenes have been replaced by pBR322 (shown as a wavy line). Only the *nos* gene (indicated in black) as well as the T-DNA border terminal sequences (indicated by jagged lines) of the T-DNA region remain. A foreign gene of interest cloned in a pBR-type plasmid can be inserted into pGV3850 by a single recombination event through the homologous pBR sequences. The hatched triangle indicates an additional antibiotic resistance marker gene other than ampicillin resistance in order to select for the recombination event. Recombination between homologous pBR regions results in the relative reversal of the foreign gene in the cointegrate structure seen below.

regenerates retain the introduced marker functions, the top shoots of these plants were transferred to medium containing 100 μg of kanamycin per milliliter. They were able to form roots and maintain normal growth. In contrast, the top shoots of normal tobacco plants are not capable of root formation, and eventually die on kanamycin-containing medium.

Prospectives

The experiments presented here not only emphasize the effectiveness of the Ti plasmid as a vector for transfer of foreign genes to plants but also its potential to study the expression and regulation of the transferred genes. Future experiments will lead to a better understanding of how the T-DNA is transferred as well as how it carries out its biological effects. These studies will undoubtedly allow the design of improved Ti plasmid vectors.

For example, there is much interest in a binary system consisting of a modified T-DNA on one plasmid and the Ti-specific functions of the *vir* region on another. Initial experiments have shown that this system can transfer the oncogenic functions of the T-DNA region to plants (*30*). It is now necessary to show that a nononcogenic T-DNA derivative containing only the border regions can also be transferred efficiently to plant cells. The drawback of this system is that a wide host range replicon must be used in order to maintain the T-DNA as a separate plasmid in both *E. coli* (where cloning can be done easily) and *Agrobacterium*. Such replicons are often large and contain multiple restriction sites; a functional T-DNA–replicon vehicle should be as small as possible to provide unique restriction sites suitable for cloning DNA.

Although we have limited our discussion of DNA vectors to use of the Ti plasmid system, there are other possible candidates. Cauliflower mosaic virus (*65*) has been studied extensively since it can infect leaves in situ and later move systematically throughout the entire plant. This system is surely limited both in its host range and in its ability to transfer more than 500 bp of foreign DNA (*66*), but it may be of more practical value as a source of DNA regulatory sequences in constructions to be transferred and expressed in plants. Geminiviruses (*67*) have recently attracted attention since they are pathogens of a wide variety of plants including legumes and cereals and thus are potential candidates to introduce genes into monocotyledonous plants. However, the basic biology of geminiviruses is still being investigated; the genome of such a virus was cloned recently in *E. coli* and its sequence is being determined.

Another possible vector system is modeled on the recent successful use of transposable elements containing foreign DNA as carriers to introduce DNA into *Drosophila* (*68*). P-elements containing DNA inserts can be injected into *Drosophila* embryos where they integrate at random and are expressed in a tissue-specific developmentally regulated manner. Analogous types of experiments may be possible in plants. There is extensive biological information available on the "behavior" of plant movable elements (*69*). The Ac/Ds "controlling elements" of corn in particular look promising for adaptation as gene vectors and some of these elements have recently been cloned (*70*).

It is clear that DNA transfer to plants can be achieved by using existing techniques, and the number of possible vectors will probably increase in the future. We have demonstrated that the Ti plasmid can be used as an effective acceptor plasmid for any foreign DNA sequence, and that plant cells transformed with such a vector are fully capable of growth and differentiation. Thus we can now begin to study aspects of plant biology that are necessary both for basic knowledge and for more applied research. The totipotency of plant cells, in addition to their ability to grow under various environmental conditions, makes them particularly interesting subjects. The successful use of DNA transfer vectors for plants is dependent on advances in two major areas of plant research, namely (i) the isolation of particularly interesting genes and an analysis of their control; and (ii) the improvement of plant tissue culture techniques that will make it possible to study more agronomically important species.

Major efforts will be directed toward the isolation of plant genes that provide fundamental information. For example, an analysis of genes regulated by light is necessary to answer questions basic to the physiology of plants; our experiments indicate that the control regions of such genes are readily amenable to analysis. Additional studies of isolated genes will help define what is required for the transport of gene products to different organelles or for conferring resistance to pathogens or environmental stress.

To date, the major hosts for Ti-mediated DNA transfer experiments have been various species of *Nicotiana* (for example, tobacco). Potatoes (*71*), carrots (*72*), and flax (*73*) have been used to a lesser extent. *Agrobacterium* is known to infect many species throughout the whole spectrum of dicotyledonous plants, although few are amenable to tissue culture techniques. There has been significant progress in the culture of several of the *Brassica* (for example, rapeseed) (*74*) and *Solanum* (for example, eggplant) (*75*); however, many other important crop plants, such as the legumes, alfalfa, and soybean cannot yet be propagated easily in tissue culture.

There is no DNA transfer system available for monocotyledonous plants, which include the important cereal food crops. Thus far, the monocotyledons have been resistant to infection by the Ti system of *Agrobacterium*. The barrier to infection may be either the transfer of DNA or the inability of the infected cells to respond in a tumorous fashion. The use of nononcogenic Ti plasmid vectors in combination with new selectable marker genes may eventually help to solve this problem.

In conclusion, the availability of recombinant DNA techniques and the discovery of the Ti plasmid system have opened the plant kingdom to gene transfer experiments. We expect that the techniques outlined here will help to elucidate interesting biological pathways that are unique to plants.

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