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Bacterial Plasmids as Natural Vectors for Plants

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INTRODUCTION

The development of experimental procedures for the genetic transformation of plant cells is a basic step concerning plant genetic engineering. The following approaches have or could be used to introduce genes into eukaryotic cells.

Direct uptake of DNA from solution or after coprecipitation with calcium phosphate. Despite the large amount of literature concerning the use of these methods with plant cells and protoplasts, most, if not all, of this work is inconclusive because of the lack of direct evidence for stable uptake of the transforming DNA, e.g., by DNA/DNA hybridizations.

Micro-injection. The direct injection of DNA in the nuclei of animal cells has been developed over the last years into an extraordinarily potent tool. No successful attempt to use this method for plant cells has yet been reported.

DNA viruses as host gene vectors. Only very few plant DNA viruses are known. The best studied virus is CaMV, which infects some crucifer plants. The nucleotide sequence has been worked out by Franck et al. (1980) and work is in progress to try to develop this virus genome as a gene vector. The most important drawback of its use as a gene vector at present is the relatively narrow host range and the fact that only very small DNA fragments of up to about 300 bp can be stably inserted into the CaMV genome. For a recent review, see Hohn and Hohn (1982).

The Ti plasmids of A. tumefaciens. For dicotyledonous plants this natural gene vector has been successfully used to experimentally introduce isolated genes into the chromosomes of plant cells. A review of the properties of Ti plasmids as gene vectors will therefore be presented in this paper.

DISCUSSION

The Ti Plasmid as a General Gene Vector

Crown gall is a neoplastic disease in plants. The causative agents of these plant

tumors are a group of gram-negative soil bacteria, belonging to the genus *Agrobacterium.* Upon wounding and infection of plants, agrobacteria can transform plant cells into tumor cells proliferating autonomously in tissue culture on simple media devoid of growth hormones. The disease is very widespread in nature, affecting most dicotyledonous plants. Crown gall formation on monocotyledonous plants has never been convincingly documented (De Cleene and De Ley 1976).

Crown gall cells, as a direct result of genetic transformation by Ti plasmids, produce various substances called opines. Free-living agrobacteria utilize these opines as sources of energy, carbon, and nitrogen. All tumor-inducing *A. tumefaciens* strains studied thus far have been shown to contain large extrachromosomal, covalently closed, circular DNA plasmids (Zaenen et al. 1974). Plasmid elimination (Van Larebeke et al. 1974; Schell 1975) and plasmid transfers from oncogenic strains to nononcogenic, plasmid-free receptor strains (Van Larebeke et al. 1975; Watson et al. 1975) demonstrated that these plasmids are the causative agents for tumor induction. These plasmids were therefore called Ti (Tumor-inducing) plasmids. In addition, it was shown that the specificity of opine synthesis in transformed plant cells as well as the catabolism of specific opines are directly coded for by the type of Ti plasmid present in the transforming *Agrobacterium* strain (Bomhoff et al. 1976). Thus, according to the type of opine made in the plant tumor cell, the Ti plasmids can be grouped into octopine, nopaline, and agropine classes (Guyon et al. 1980).

The molecular basis underlying the transformation of the plant cell into a tumorous state as well as the synthesis of the opines in the transformed plant cell is the transfer integration and stable maintenance of a well-defined part of the Ti plasmid (called the T-region) into the nuclear genome of the plant cell.

Thus, with the advent of Ti plasmids, agrobacteria have developed a new type of parasitism: plant cells are forced genetically (via a gene transfer mechanism) to produce specific compounds (opines) which agrobacteria-harboring Ti plasmids are uniquely equipped to catabolize. This novel type of parasitism has, therefore, been called "genetic colonization" (Schell et al. 1979).

The Integration of the T-region of Ti Plasmids in Chromosomal Plant DNA

The transfer and stable maintenance of the T-region of Ti plasmid in plant cells result from its integration in nuclear DNA. This was studied in detail by Southern blotting analysis, and more recently by reisolation of junction fragments linking T-DNA to plant DNA (Chilton et al. 1980; Lemmers et al. 1980; Thomashow et al. 1980; Zambryski et al. 1980; Willmitzer et al. 1980; De Beuckeleer et al. 1981). The results show that all crown-gall tumors contain a DNA segment, called the T-DNA, which is homologous to DNA sequences in the Ti plasmid used to induce the tumor line. In all cases, this T-DNA corresponds to, and is colinear with, a continuous stretch of Ti plasmid DNA, which has therefore been called the T-region. The T-region of octopine and nopaline Ti plasmids has been studied in most detail, both physically and functionally. The T-regions, roughly 23 kb in size, are only a portion of the entire plasmids. The T-DNA segment present in different tumors induced on tobacco by the nopaline strains C58 and T37 was found to be almost identical. It had in each case a size of about 23 kb and represented one contiguous fragment of the Ti plasmid

(Lemmers et al. 1980; Zambryski et al. 1980).

The situation is a little more complex in the case of octopine tumors. Thus, a left T-DNA region (TL-DNA) is always present; this region is usually 12 kb in size but one *Petunia* tumor line is shortened at the right end of TL by about 4 kb (De Beuckeleer et al. 1981). In addition, there is often a right T-DNA region (TR) which contains sequences which are adjacent to, but not contiguous with, TL-DNA in the octopine Ti plasmid, and in one tumor line TR is amplfied (Merlo et al. 1980; Thomashow et al. 1980). The T-DNA's of nopaline and octopine tumor lines are not identical. However, Southern blotting and cross-hybridization of restriction endonuclease digests of the two types of plasmids as well as electron microscope heteroduplex analysis have revealed that 8 to 9 kb of the T-DNA regions are conserved (Depicker et al. 1978; Chilton et al. 1978; Engler et al. 1981); these sequences are represented as the shaded areas in Fig. 1. The information available up to now is

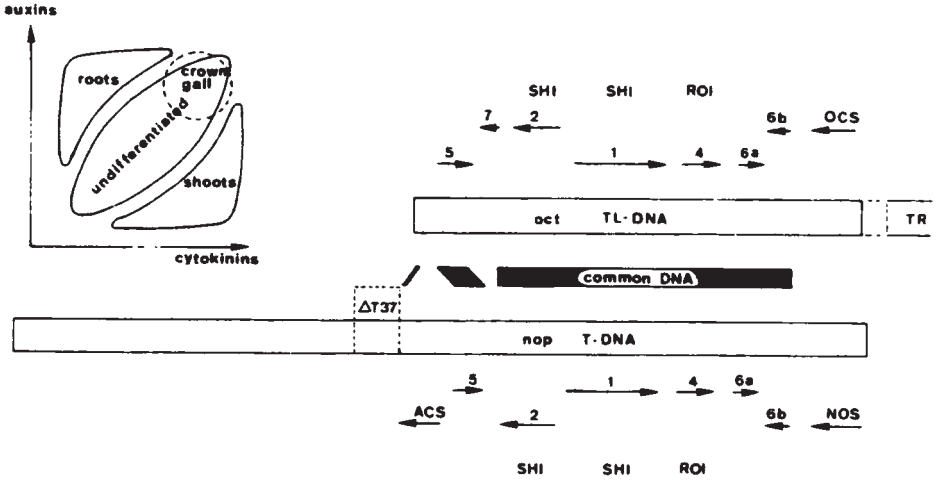

FIG. 1. The octopine and nopaline T-DNA map with their localized transcripts. Their homology (Engler et al. 1981) is indicated as a bar between both T-DNA's with the gene they specify. The eight transcripts are mapped on the octopine T-DNA according to Willmitzer et al. (1982b). Only the six nopaline T-DNA transcripts, homologous with the octopine T-DNA, are indicated; the other nopaline T-DNA transcripts are presented elsewhere (Willmitzer et al. 1983). Shi (shoot inhibition), Roi (root inhibition), Ocs (octopine synthase), and Nos (nopaline synthase) are phenotypes corresponding to the mapped loci on the T-DNA. The auxin-cytokinin diagram is a schematic representation of how the hormone levels can influence the callus morphology (Skoog and Miller 1957). Variations of the auxin-cytokinin ratio cause the callus to give rise to buds, shoots, or roots. A similar control could be exerted by the T-DNA in a direct or indirect way. Inactivation of T-DNA transcripts can cause a different crown-gall morphology possibly as an alteration in the auxin-cytokinin ratio.

not sufficient to elaborate a model for the integration of the T-DNA. The sequences of the left and right border regions of the nopaline Ti plasmid have been determined (Zambryski et al. 1980, 1982). The data from the exact nucleotide sequence of four right and four left T-DNA borders isolated from nopaline tumor lines suggest that the mechanism of T-DNA integration, and subsequent stabilization, is precise on the right and less precise on the left. The T-DNA/plant DNA junction in the right has been shown to occur at exactly the same base pair with respect to Ti plasmid sequenc-

es in the T-DNA isolated from two independent tumor lines. Only a variation of a single base was found in the analysis of the right border of a junctional region containing the left-right T-DNA boundary from a T-DNA which was part of a tandem array. All the left T-DNA boundaries determined so far are different.

In addition, the sequences around both the right T-DNA border region and the left T-DNA border region of the nopaline Ti plasmid were compared. There is a short direct repeat of 12 base pairs which can be extended to 25 base pairs with three mismatched bases, which is found on either side of the T-region of the nopaline Ti plasmid. The 25 base pairs have the following sequence:

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                    G C   G
T G G C A G G A T A T A T T G T G G T G T A A A C

A C C G T C C T A T A T A A C A C C A C A T T T G
                    C G   C
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This sequence is found just one base pair to the right of the T-DNA border on the right side of the nopaline Ti plasmid and 100 base pairs to the left of the left T-DNA border on the left side of the nopaline Ti plasmid. A very similar sequence also has been observed near the left and the right borders of the octopine TL-region (Holsters et al. 1983).

Demonstration of the Cotransfer to Plant of DNA Segments Inserted in the T-DNA

The fact that the integration of the T-DNA fragment is possibly directed by specific sequences bordering the T-region is of particular interest for its use as an experimental gene vector. Indeed, one could expect that any DNA sequence, integrated inbetween these recognition sequences, will be cotransferred to the plant DNA. This was first shown to be the case by inserting a bacterial transposon (Tn*7*, 9.2 Md) in the T-region of a nopaline Ti plasmid, and demonstrating that the plant tumors induced by this plasmid contained a complete and intact Tn*7* sequence inserted in the T-DNA sequence (Hernalsteens et al. 1980). That the Tn*7* inserted in the plant DNA was not subjected to sequence rearrangements was demonstrated by recloning the Tn*7* from the plant DNA. The recombinant cosmids containing Tn*7* DNA covalently linked to plant DNA were identified by directly selecting for the expression of Tn*7* (streptomycin, spectinomycin, trimethoprim resistance) (Holsters et al. 1982).

These observations have, therefore, firmly established that the Ti plasmids can be used as experimental gene vectors and that large DNA sequences (of up to 50 kb) can be stably transferred to the nucleus of plant cells as a single DNA segment.

Expression of T-DNA in Transformed Plant Tissues

Previous work has shown that the T-DNA insert is transcribed in transformed plant cells (Drummond et al. 1977; Gurley et al. 1979; Willmitzer et al. 1981a; Gelvin et al. 1981), but the number, sizes, and location of the coding regions were not known. Recently, these questions were investigated in detail (Willmitzer et al. 1982, 1983). Cell-suspension cultures from tobacco used in these experiments harbor the T-DNA

from pTiA6, a Ti plasmid which induces octopine synthesis in transformed cells. The cells contain only the TL fragment of the T-region (Fig. 1). Tumor-specific RNA's were detected and mapped by hybridization of ^{32}P-labeled Ti plasmid fragments to polyadenylated RNA which had been separated on agarose gels, and then transferred to DBM paper. The results summarized in Fig. 1 show that the cells contain a total of eight distinct transcripts which differ in their relative abundance and in their sizes. They all bind to oligo(dT)-cellulose, indicating that they are polyadenylated. Thus, the T-DNA which was transferred from a prokaryotic organism must provide specific poly(A) addition sites. The direction of transcription was determined for six of the eight transcripts, and the location of the approximate 5'- and 3'-ends were mapped on the TL-DNA.

All eight RNA's mapped within the T-DNA sequence. This, and the observation that transcription is inhibited by low concentrations of α-amanitin (Willmitzer et al. 1981b), seems to suggest that each transcript is determined by a specific promotor site on the TL-DNA recognized by plant RNA polymerase II. These data do not rule out that some T-DNA promoters serve for transcription of more than one RNA. Considering the groupwise orientation of several transcripts (Fig. 1), the simplest model would assume one promoter site per group of transcripts. If so, one would expect that inactivation of a 5'-proximal gene of a group would lead also to disappearance of the transcripts from the 5'-distal genes. However, analysis of some cell lines containing the T-DNA of Ti plasmid mutants indicates that groupwise inactivation of genes did not occur (Leemans et al. 1982). The results available so far are consistent with the assumption that each gene of TL-DNA has its own signals for transcription in the eukaryotic plant cells. A similar conclusion also was reached for the transcripts encoded by the nopaline T-DNA (Willmitzer et al. 1983), and for the transcripts encoded by TR-DNA in octopine tumors (Velten et al. in preparation).

The T-DNA Contains Transcription Promoter Signals

The 5'-end of both the nopaline synthase as well as the octopine synthase mRNA were mapped accurately by sequencing of a T-region DNA fragment hybridized to the mRNA, and thus protected from degradation by the single strand-specific S1 nuclease (Depicker et al. 1982; De Greve et al. 1982a). In addition, the complete nucleotide sequence from the octopine as well as from the nopaline synthase gene was determined. The promoter sequences that have thus been identified are more eukaryotic than prokaryotic in their recognition signals, and no introns interrupt the open-reading frame which starts at the first AUG codon following the 5'-start of the transcript. Recent experiments in our laboratory demonstrate that these promoters can be used to express foreign genes in plants, such as the neomycin transphosphorylase of Tn5 or the methotrexate-resistant dihydrofolate reductase of Tn7 (Herrera-Estrella et al., in preparation).

Genetic Identification of Tumor-Controlling Genes

A detailed genetic analysis of the TL-region of octopine crown galls (Leemans et al. 1982) when correlated with an analysis of the number, size, and map position of TL-

DNA-derived polyadenylated transcripts (Willmitzer et al. 1982), has allowed us to assign functions to some of the eight different well-defined transcripts, coded for by TL-DNA.

The two most abundant transcripts, i.e., transcripts 7 and 3 of respectively 670 and 1,400 nucleotides, are specific for octopine tumors. Transcript 3 was found to code for the enzyme LpDH which synthesizes octopine (Schroder et al. 1981). The functions of transcript 7 is still unknown. The other six transcripts are derived from TL-DNA sequences that are homologous with an equivalent region in the T-DNA of nopaline tumors (Fig. 1).

Transcripts 1, 2, 4, 5, 6a, and 6b have homologous counterparts of the same size in nopaline tumors (Willmitzer et al. 1983). By observing the properties of crown-gall tumors obtained by infection of tobacco with mutant octopine or nopaline Ti plasmids carrying deletions of specified segments of the TL-region, it was possible to assign functions to transcripts 1, 2, and 4, and to suggest a possible function for transcript 5 (Leemans et al. 1982).

The main conclusions from these studies were: (1) most, if not all, of the transcripts were expressed from individual promoters; (2) T-DNA transfer and tumorous growth are controlled by different and independently acting functions; (3) none of the T-DNA-derived transcripts are essential for T-DNA transfer; (4) transcripts 1, 2, 4, and probably also 5, act by suppressing organ development; (5) shoot formation (transcripts 1 and 2) and root formation (transcript 4) are suppressed by the action of different transcripts; (6) transcripts 4 and 6 are sufficient to maintain the tumorous growth properties of transformed tobacco cells; (7) transcripts 1 and 2 stimulate root formation in the absence of transcript 4, and reciprocally transcript 4 stimulates shoot formation in the absence of transcripts 1 and 2.

Regeneration of Normal Plants From T-DNA Containing Plant Cells

The ultimate aim of many experimental gene transfer attempts in plants is to produce fertile cultivars harboring and transmitting new genetic properties. It was, therefore, essential to determine whether T-DNA transfer could be dissociated from neoplastic transformation and whether normal, fertile plants could be derived from T-DNA-transformed plant cells. If the conclusions drawn from the genetic identification of tumor-controlling genes are correct, it follows that elimination of the coding sequences for transcripts 1, 2, 4, and 5, from the T-DNA of transformed cells should result in tobacco cells containing and expressing some of the T-DNA genes, e.g., transcript 3 (thus producing LpDH activity), but no longer suppressed for organ development. By starting with a tumor line induced by a plasmid where transcript 2 was inactivated by a Tn*7* insertion (pGV2100), such plants could be obtained (De Greve et al. 1982b). These plants were shown to contain lysopine dehydrogenase activity, and polysomal RNA was found to contain T-DNA transcripts homologous to the opine synthesis locus (transcript 3). No other transcripts derived from the T-region were observed.

These observations demonstrate that normal plants can be obtained from plant cells transformed with Ti plasmids genetically altered in specific segments of the T-region.

Mendelian Inheritance of T-DNA Linked Genes

A number of published observations (Braun and Wood 1976; Yang et al. 1980) had been interpreted to indicate that T-DNA sequences in grafted teratomas were lost during meiosis in flowering grafts. This led to the important suggestion that T-DNA sequences might be excised during meiosis.

To test this possibility sexual crosses were performed using the flowering tobacco plant rGV1 containing a partially deleted T-DNA, and expressing transcript 3 (see above). It was found that the T-DNA was stably transmitted through meiosis, both through the pollen and through the eggs, and segregated as a single dominant Mendelian gene (Otten et al. 1981) (Table 1).

TABLE 1. *Transmission of the octopine synthase gene in tobacco plant rGV1: the gene is transmitted to progeny as a dominant Mendelian gene*

Crosses	No. of Progeny Tested	Octopine Synthesis	
rGV1 x rGV1	145	110 (76%)	35 (24%)
rGV1 x rGV1	200	++[a] 42 (21%)	+[a] 95 (48%) – 63 (31%)
rGV1 x wild-type	248	124 (50%)	124 (50%)
rGV1 x wild-type	187	81 (43%)	106 (57%)
Plantlets derived from anther cultures (haploid)	102	47 (46%)	55 (54%)

[a]As estimated from a semi-quantitative assay of enzyme activity.

CONCLUSIONS

With the crown-gall system, we are confronted with a well-documented, natural instance of a translocation system involving DNA transfer between prokaryotes and eukaryotes. The T-DNA is a translocating element present in a prokaryotic plasmid, but consisting of DNA sequences which become functional genes in eukaryotic cells.

It is interesting to speculate that other such natural instances of DNA translocations, involving widely different organisms, can be expected to occur in situations involving a close and long-term symbiotic or parasitic association between these different organisms.

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