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TAILORING THE AGROBACTERIUM Ti PLASMID AS A VECTOR FOR PLANT GENETIC ENGINEERING

(site specific insertions, chimeric gene construction, regeneration of plants containing T-DNA)

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SUMMARY

Agrobacterium tumefaciens *and its tumor-inducing (Ti) plasmids constitute a promising vector system for the introduction of desirable genes into higher plant cells. This bacterial pathogen has an unusually wide host range, inciting galls on many kinds of dicotyledonous plants. By a mechanism as yet unknown, during tumor induction a specific part of the Ti plasmid becomes covalently joined to plant nuclear DNA. This foreign DNA, called T-DNA, is stably maintained and expressed in the transformed plant cell. There are several technical barriers to be overcome if we are to exploit the Ti plasmid as a vector for introducing new genes into plants. First is the problem of inserting specific desired genes into the T-DNA portion of the Ti plasmid. Second is how to induce or regulate the expression of foreign genes in the new host plant. Third is the challenge of regenerating healthy plants containing T-DNA. Progress in each of these areas is encouraging. The most difficult problem remaining will be the choice and isolation of genes (as genomic or cDNA clones) whose introduction into plants will produce agronomically desirable changes.*

INTRODUCTION

The plant cancer crown gall is caused by a class of tumor-inducing (Ti) plasmids of the common soil bacterium *Agrobacterium tumefaciens* (ZAENEN et al. 1974, VAN LAREBEKE et al. 1974, WATSON et al. 1975). Tumor tissue cultivated free from the bacterial pathogen retains the transformed phenotype, i.e. it grows luxuriantly in vitro without exogenous phytohormones (BRAUN 1947, BRAUN & MANDLE 1948) and produces

novel matabolites now called opines, whose synthesis is directed by genetic information on the Ti plasmid (PETIT et al. 1970, BOMHOFF et al. 1976, MONTOYA et al. 1977, KLAPWIJK et al., 1976, HERNALSTEENS et al. 1980, GUYON et al. 1980). The molecular basis of this transformation is now unfolding in considerable detail. The tumor-inducing principle first postulated by Braun (BRAUN 1947) has been identified as a specific portion of the Ti plasmids called T-DNA that becomes incorporated into the nuclear genome of transformed plant cells (CHILTON et al. 1977, MERLO et al. 1980, YANG et al., 1980, LEMMERS et al. 1980, THOMASHOW et al. 1980a,b, YADAV et al. 1980, ZAMBRYSKI et al. 1980, WILLMITZER et al. 1980, CHILTON et al. 1980). T-DNA of diverse types of Ti plasmids includes a highly conserved region that is presumed to code for common tumor-inducing functions (DEPICKER et al. 1978, CHILTON et al. 1978). T-DNA transcripts (DRUMMOND et al. 1977) are produced in tumor nuclei (WILLMITZER et al. 1980) by an RNA polymerase that is sensitive to 0.7 μg/ml α-amanitin, presumably RNA polymerase II (SCHELL et al. 1981). The transcripts are polyadenylated (LEDEBOER 1978) and are found on polysomes (MCPHERSON et al. 1980, YANG et al. 1980). The complexity of the pattern of transcription suggests that T-DNA must contain promoters that function in the plant nucleus (GURLEY et al. 1979, GELVIN et al. 1981).

Thus the prokaryotic Ti plasmids contain genes that function in a eukaryotic plant cell. The biological rationale for this intriguing phenomenon is that the Ti plasmid acts as a natural genetic engineering vector. The novel metabolites synthesized by crown gall tumors are specific catabolic substrates for *Agrobacterium* strains possessing Ti plasmids and the Ti-plasmid-encoded catabolic enzymes are specifically induced by the corresponding opines (PETIT et al. 1970, KLAPWIJK et al. 1977,1978, PETIT & TEMPE 1978, KLAPWIJK & SCHILPEROORT 1979, ELLIS et al. 1979, ELLIS 1980). Interbacterial conjugation of Ti plasmids is also induced by opines: octopine induces transfer of the octopine-type Ti plasmid (GENETELLO et al. 1977, KERR et al. 1977), and agrocinopine induces conjugation of the nopaline-type and agropine-type Ti plasmids (ELLIS 1980). Because plasmid transfer from virulent to avirulent *Agrobacterium* strains was first noted to occur in primary crown galls on plants (KERR 1969, 1971), it seems clear that opine synthesis by tumor cells creates a unique ecological niche favorable to the pathogen.

While opine synthesis has presumably played a central role in the evolution of Ti plasmids, it is not *per se* essential to the transformation of the plant cells. A Tn7 insertion mutation in pTi C58 located near the right edge of T-DNA has been found to annihilate nopaline synthesis by tumors without affecting ability of this Ti plasmid to incite tumors (HERNALSTEENS et al. 1980). There is also evidence that the tumorous state is not essential to synthesis of nopaline: BT37 tumor tissue upon grafting yields normal-appearing leaves, stems and flower parts in which the tumorous phenotype is suppressed but nopaline synthesis continues (BRAUN & WOOD, 1976, TURGEON et al. 1976, WOOD et al. 1978, BINNS et al. 1981). While the genetic information responsible for octopine and nopaline synthesis is encoded by the Ti plasmid and thus present in Agrobacterium, these opines are not synthesized by

the bacterium, for the catabolic enzymes remain uninduced when the bacteria are cultivated in common bacteriological media (PETIT & TEMPE 1978, KLAPWIJK et al. 1978).

SITE-SPECIFIC INSERTION OF FOREIGN DNA INTO T-DNA

Comparison of T-DNA on the Ti plasmid with T-DNA in the plant tumor by Southern blotting analysis has revealed that the part of the Ti plasmid maintained in the plant cell can vary greatly for octopine tumors (MERLO et al. 1980, THOMASHOW et al. 1980a) but is nearly constant for nopaline tumors (LEMMERS et al. 1980). For the exploitation of T-DNA as a vector, one must insert passenger DNA at a position on the Ti plasmid that is always a part of T-DNA. In addition, one must choose a site that does not damage the mechanism (if any) by which T-DNA inserts into the host plant genome.

The technique used for site-specific insertion of foreign DNA into T-DNA is indirect, for the large size of T-DNA precludes its direct use as a cloning vector. Known T-DNA fragments are subcloned into *E. coli* cloning vector plasmids. A cloned T-DNA fragment is then opened at a unique central restriction endonuclease cleavage site, and into this site passenger DNA, including a selectable genetic marker, is ligated (MATZKE & CHILTON 1981, LEEMANS et al. 1981). The entire plasmid is then recloned into a wide host range plasmid for introduction into *Agrobacterium* (MATZKE & CHILTON 1981, LEEMANS et al. 1981) (Figure 1). In one version of this strategy, the wide host range plasmid serves as the passenger DNA, thus eliminating one step in the procedure (LEEMANS et al. 1981). The final step is the isolation of bacteria in which double recombination has replaced the wild type fragment on the Ti plasmid by the "engineered" fragment on the recombinant plasmid. This can be achieved by using plasmid incompatibility to select against the recombinant plasmid while maintaining positive selection for the passenger trait (MATZKE & CHILTON 1981) (Figure 1). Alternatively, recombinants can be found by screening Ti-plasmid-containing transconjugants for presence of the passenger trait and absence of a second marker on the recombinant plasmid (LEEMANS et al. 1981).

The techniques described above have been used to introduce passenger DNA at specific restriction endonuclease cleavage sites (MATZKE & CHILTON 1981, LEEMANS et al. 1981), or to replace whole T-DNA fragments or groups of fragments by passenger DNA (insertion/deletion) (LEEMANS et al. 1981). A minor variation on one of the schemes is the use of transposon mutagenesis to insert new DNA at various positions in a T-DNA clone (GARFINKEL et al. 1981). The latter approach allows access to all parts of T-DNA, irrespective of the restriction endonuclease cleavage sites, and has produced a detailed functional map of octopine T-DNA (GARFINKEL et al. 1981). Taken together with other functional mapping studies achieved by random transposon mutagenesis (HOLSTERS et al. 1980, GARFINKEL & NESTER 1980, OOMS et al. 1980, 1981, DE GREVE et al. 1981), the detailed map of Garfinkel et al. shows that no single insertion in T-DNA renders it completely avirulent. Two insertions scored as avirulent in an earlier study

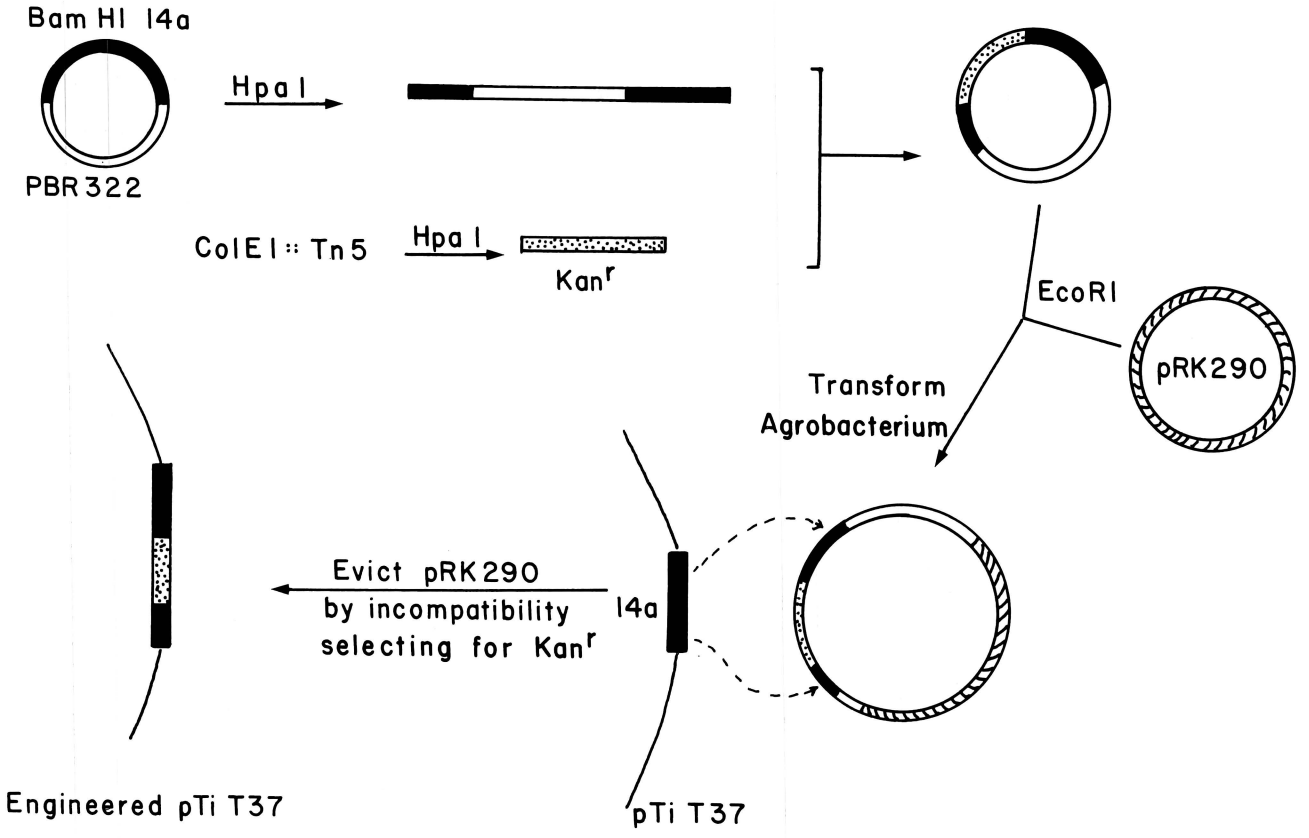

Figure 1. Scheme for site-specific insertion of DNA into T-DNA of the Ti plasmid. The method of Matzke & Chilton (1981) is illustrated here. The "eviction" step employs P1-type plasmid R751-pMG2 which encodes gentamycin resistance.

were RP4 cointegrations (HOLSTERS et al. 1980), whose effect may be ascribable to the enormous physical size of the insert. Insertion of blocks of foreign DNA of modest size (9-12 Md) (HERNALSTEENS et al. 1980, MATZKE & CHILTON 1981) does not interfere with the ability of T-DNA to insert itself into the plant genome. Further, mutations at many positions in T-DNA have failed to inactivate T-DNA insertion mechanisms, suggesting that any genes involved must map outside T-DNA, and that any structures involved must be small. These characteristics make T-DNA a more promising vector than cauliflower mosaic virus (CaMV), whose viability is highly sensitive to the size and position of foreign DNA inserts (HOWELL et al. 1981). T-DNA is biologically different from viruses in that it has evolved as a gene vector, not as a viable entity containing genes essential for its survival.

EXPRESSION OF FOREIGN GENES

The techniques described above have been used to insert several prokaryotic and eukaryotic genes into the T-DNA of various Ti plasmids, and tumor lines have been incited with the resulting *Agrobacterium* strains. In our laboratory, we have inserted the kanamycin resistance determinant from Tn5 (MATZKE & CHILTON 1981), a genomic clone of yeast alcohol dehydrogenase-I (WILLIAMSON et al. 1980, BARTON, unpublished data), and a genomic clone of maize zein (LEWIS et al. 1981, MATZKE, unpublished data). Thus far we have detected no protein product from these genes in the corresponding crown gall tumor lines (BARTON, unpublished data; MATZKE, unpublished data). While negative results are not definitive, it seems clear that the engineered traits are not expressing in our crown gall tumor lines at high levels.

Since T-DNA genes to which our engineered traits are attached do express in the engineered tumor lines (which produce nopaline), we infer that the passenger genes fail to express efficiently in the plant because their structure is foreign. Although there are features of eukaryotic gene structure and regulation that remain mysterious, certain features are recognized as important: a promoter region 5' to the start of transcription that allows RNA polymerase to bind to the DNA, and consensus sequences at -70 and -30 bases from the start of transcription (GROSVELD et al. 1982), as well as signals for termination and polyadenylation at the 3' end of the gene (EFSTRATIADIS et al. 1980). The length and sequence of the 5'-untranslated portion of the transcript could affect the efficiency of capping, splicing, transport to the cytoplasm or initiation of translation. Structures of the 5' and 3' ends of the mature mRNA could influence its stability in the nucleus and/or cytoplasm. Thus there are many potential problems for foreign gene expression.

An approach to this complex problem that should circumvent many of the barriers is the construction of "chimeric genes" containing the coding region of the passenger gene and the non-coding signals of a gene that expresses in plants (Figure 2). We are working on such chimeric gene constructions, using the signals of nopaline synthase, a T-DNA gene that expresses constitutively in plant cells. For this

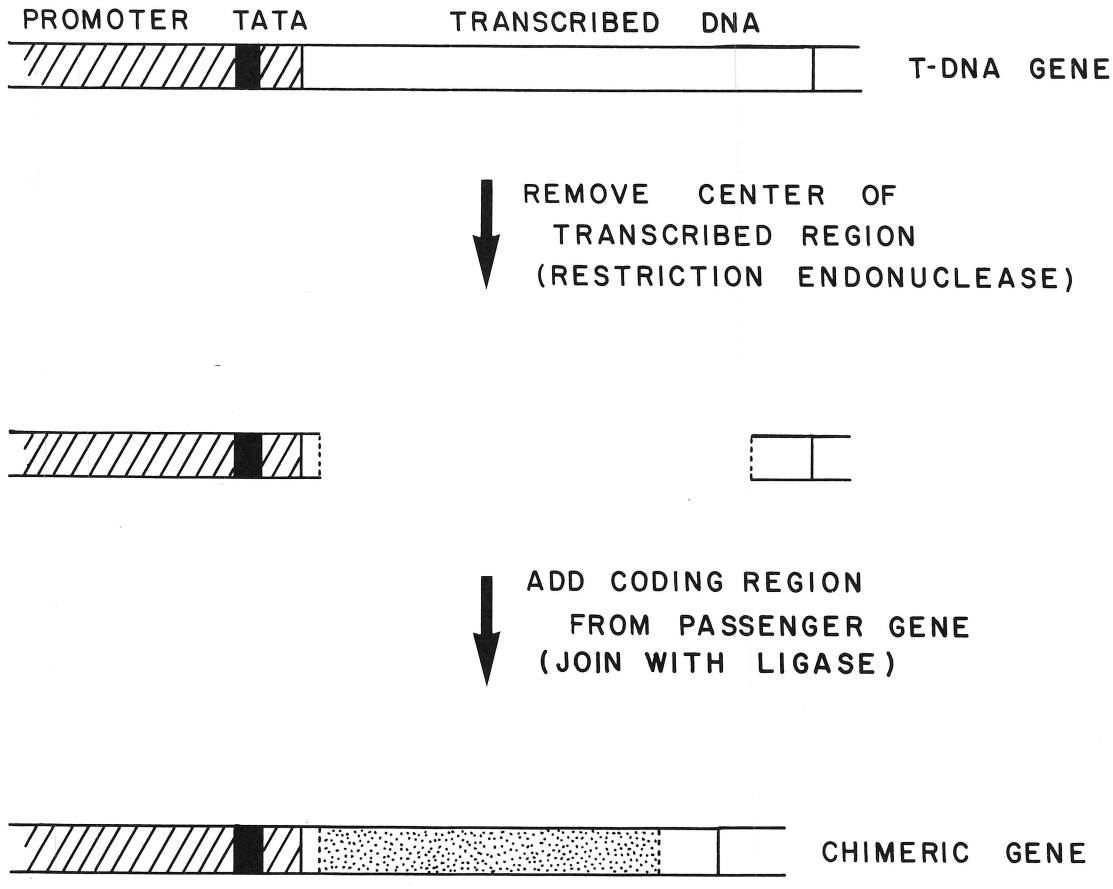

Figure 2. Scheme for construction of chimeric gene using signal portions of a T-DNA gene.

purpose we have completely sequenced the nopaline synthase gene and by S1 nuclease mapping we have identified the 5' and 3' ends of the transcript it encodes in the plant tumor. The nopaline synthase gene contains sequences similar to the consensus sequences in eukaryotic genes (GROSVELD et al. 1982, EFSTRATIADIS et al. 1980, BEVAN & BARNES, unpublished data). It does not contain canonical prokaryotic promoter sequences and is therefore a eukaryotic gene by both functional and structural criteria. The success of chimeric genes as a means for effecting gene expression in plants remains to be seen. Similar experiments in animal systems have produced a chimeric gene that is not only expressed but regulated in a predictable way (BRINSTER et al. 1981).

REGENERATION OF HEALTHY PLANTS CONTAINING T-DNA

Grafting has produced normal-appearing shoots from cloned nopaline tobacco teratomata (BRAUN & WOOD 1976, TURGEON et al. 1976, BINNS et al. 1981), but these shoots are abnormal in that they fail to produce roots. The shoots contain nopaline (BRAUN & WOOD 1976, TURGEON et al. 1976, WOOD et al. 1978, BINNS et al. 1981) and T-DNA (YANG et al. 1980, LEMMERS et al. 1980); however the progeny of such shoots appear completely normal and lack nopaline (BRAUN & WOOD 1976, TURGEON et al. 1976, BINNS et al. 1981) These findings suggested that nopaline T-DNA could not survive meiosis. More recently, normal plants were obtained as spontaneous regenerants from the same cloned nopaline tobacco teratoma, and these plants, while nopaline negative, were found by Southern blotting analysis to contain left and right border fragments of T-DNA (YANG & SIMPSON, 1981). Progeny of these plants exhibited the same T-DNA border fragments (YANG & SIMPSON 1981), proving that T-DNA borders, at least, could be transmitted through gametes. These two phenomena, taken together, suggest that genetic information encoded by the central region of T-DNA is incompatible with normal plant development. Thus if T-DNA were "disarmed" by excision or inactivation of appropriate T-DNA genes, presumably one could obtain normal plant cells containing T-DNA, and from these, normal plants.

Transformed tobacco cells derived from infection of regenerating protoplasts have been found to produce opine positive graftable suppressed shoots (WULLEMS et al. 1981a,b) whose F_1 progeny in most instances are opine negative. However a total of nine nopaline positive progeny were obtained, and even among opine negative progeny certain characteristics of transformed cells were retained (abnormal flower morphology, insusceptibility to infection by *A. tumefaciens*) (WULLEMS et al. 1981b). These results support the view that T-DNA can survive meiosis. However no morphologically normal plants containing T-DNA were produced.

Octopine Ti plasmids carrying insertion mutations in Eco RI fragments 32 or 7 (left side) incite crown gall tumors from which shoots emerge (LEEMANS et al. 1981, GARFINKEL et al. 1981, OOMS et al. 1981, DE GREVE et al. 1981, OTTEN et al. 1981). A tumor incited by such a "shooter" mutant was found to produce one octopine positive normal shoot that

developed roots and was fertile (OTTEN et al. 1981). The progeny of this normal plant segregated the octopine marker as a dominant mendelian trait (OTTEN et al. 1981). The progeny are sensitive to crown gall and morphologically normal. Additional examples of normal plants have been isolated from tumors incited by "shooter" mutant Ti plasmids (OTTEN et al. 1981); thus this appears to be a feasible route to normal plants from crown gall tumors.

Normal plants have also been regenerated from tissue transformed by the Ri (root-inducing) plasmid of *Agrobacterium rhizogenes*, agent of hairy root disease in a wide range of dicotyledonous plants (MOORE et al. 1979, WHITE & NESTER 1980a,b, CHILTON et al. 1982). The mechanism of the induction of transformed roots is similar to that of crown gall tumors: the virulence plasmid contains T-DNA that becomes inserted into the host plant genome (CHILTON et al. 1982). Transformed roots synthesize agropine and/or mannopine, opines also found in certain crown gall tumors (TEPFER & TEMPE 1981, GUYON et al. 1980, PETIT & TEMPE, unpublished data). Transformed mannopine-containing carrot root cultures were induced to pass through callus and embryogenesis to form normal-appearing plants that contain mannopine in their leaves and roots (CHILTON et al. 1982). It thus appears that the Ri plasmid is a natural example of a "disarmed" T-DNA vector. Ri plasmid transformed tissue morphologically resembles tissue transformed by "rooty" mutants of Ti plasmids. Experiments are underway in our laboratory to determine whether such mutants can indeed yield transformed plant tissue that retains the ability to regenerate whole plants.

CONCLUSIONS

Two major technical obstacles that beset the Ti plasmid as a vector have now in principle been surmounted. First, methods have been developed for introduction of desired DNA into many specific sites within T-DNA. Improvements are needed in convenience and speed of these procedures, but it is clear that this phase of the genetic engineering process can be accomplished. Second, whole plants with roots have been regenerated from transformed tissue and found to contain opines or part of T-DNA; moreover, in one case of landmark significance, the opine trait has been shown to transmit to normal F_1 progeny as a dominant mendelian trait. Although the rules governing regeneration of healthy plants from transformed tissue are not clearly discerned yet, it seems likely that this process will become reproducible and predictable as we gain understanding of the hormonal imbalance produced by various genes in T-DNA.

The next technical objective for the genetic engineer is the induction of expression of foreign genes in the engineered plant cells. Recent success of chimeric gene regulation and expression in animals (BRINSTER et al. 1981) appears to be a sound basis for optimism that similar approaches will succeed in plants. The more long range and by far most challenging problems that face the genetic engineer are the choice and isolation of genes whose

introduction into the plant genome will be of positive agricultural value. In addition, the several techniques for regeneration of whole plants that have proven successful for tobacco and carrot may prove ineffective in other kinds of plants. Thus, despite the rewarding technical progress already accomplished, we may look forward with confidence to interesting and difficult work ahead of us.

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Chilton, Burr and Merlo in informal discussion