Crown Gall Disease and Prospects for Genetic Manipulation of Plants

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Summary. *Agrobacterium tumefaciens* incites crown gall tumors when bacterial DNA integrates into plant nuclear DNA. Plant cells can express these integrated bacterial genes. Following insertion of desired genes into bacterial DNA using recombinant DNA techniques, this system permits introduction of these new genes into plant DNA. We discuss the potential for genetic manipulation of plants using *Agrobacterium tumefaciens* and the related organism *Agrobacterium rhizogenes*.

Agrobacterium tumefaciens incites crown gall tumors on many dicotyledonous plants when viable bacteria infect wounded plant tissue (*1*). Virulent *A. tumefaciens* strains harbor a large (140 to 235 kilobases) Ti (tumor-inciting) (*2*) plasmid that carries genes essential for tumorigenesis (*3, 4*). During tumor induction a specific segment of the Ti plasmid DNA called the T-DNA (transferred DNA) integrates into plant nuclear DNA (*5–10*) and is retained in axenic tumor cells during culture for several years. Crown gall tumor cells often produce opines, unusual compounds not found in normal plant cells (*11–14*) (Fig. 1). Further, unlike normal plant cells, tumor cells grow on a chemically defined medium lacking added auxins and cytokinins (plant growth hormones) (*15*); genes responsible for these new traits lie in the T-DNA. Ti plasmid mutants define: (i) genetic loci in the T region affecting tumor morphology and opine synthesis, (ii) regions outside the T region required for virulence, and (iii) regions with no apparent effect on tumorigenicity (*16–26*). A related organism, *Agrobacterium rhizogenes*, incites hairy root disease on many dicotyledonous plants (*27, 28*). A large (230 kilobases) Ri (root-inciting) plasmid harbored by virulent *A. rhizogenes* strains carries genes essential for root induction (*27*), and a segment of the Ri plasmid integrates into plant DNA (*29, 30*). Crown gall tumorigenesis and the genetic organization of the Ti plasmid have been described in reviews (*11–14, 31–35*).

General Description of Ti Plasmids

Crown gall tumor cells usually contain enzymes that synthesize either octopine family opines, nopaline family opines, agropine, or agrocinopine family opines (Fig. 1) (*12*). Some octopine-synthesizing tumors also make agropine, and agrocinopines were found in nopaline-synthesizing and agropine-synthesizing tumors (*14*). The particular opine synthase genes carried on the Ti plasmid of the inciting *A. tumefaciens* strain determine which opines the tumor makes, but the bacterium itself apparently does not express its opine synthase genes. Each opine induces synthesis of the corresponding Ti plasmid-coded opine catabolism enzymes (permease and oxidase) in the bacterium. These enzymes allow the tumor-inciting bacteria to take up and degrade the specific opine produced by the tumor. Thus, virulent *A. tumefaciens* strains use the appropriate opine as a carbon and nitrogen source.

Plasmid incompatibility and DNA sequence homology indicate plasmid relatedness. All octopine and nopaline Ti plasmids belong to the Rh-1 incompatibility group and express incompatibility functions that prevent stable maintenance of two Rh-1 plasmids in the same cell. Agropine Ti plasmids (Rh-2 incompatibility) and the Ri plasmids (Rh-3 incompatibility) are compatible with octopine and nopaline Ti plasmids and with each other in the cases tested (*14*). Ti plasmids and Ri plasmids generally share some DNA sequence homology with each other. Often, such regions of homology (within or outside the T region) code for virulence functions (*31*). Thus, there is some conservation of genes involved with virulence.

Ti plasmids carry transfer (*tra*) genes allowing transfer of a Ti plasmid between bacterial strains. Transfer and opine catabolism appear to be coordinately controlled, but the transfer and opine catabolism genes probably lie in separate operons since mutations exist that render only one function constitutive [(*33*) for review]. All currently known *tra* mutants remain virulent, and the avirulent mutants that have been tested remain transfer proficient.

Other Ti plasmid-coded functions apparently not related to virulence include exclusion of bacteriophage API (*ape*), sensitivity (Agr^S; coded by most nopaline and agropine plasmids) to the antibiotic agrocin 84 produced by *Agrobacterium radiobacter* 84, and one or more enzymes involved in arginine degradation (*arc*) (*14, 31*).

Virulence Genes Outside the T Region

A number of Ti plasmid mutations outside the T region affect oncogenicity. Physical mapping of deletion and transposon insertion mutations in octopine Ti plasmids locate genes involved in tumorigenesis (*vir* genes) in the region lying left of the T region (Fig. 2A) (*16–19, 22–23, 24*). Since other transposon insertions in this region do not affect virulence, this region probably contains several separate operons (*36*).

With two exceptions (*37*) complementation of avirulent transposon insertion mutations in the *vir* region by the corresponding wild-type region carried on another plasmid restores virulence (*38*). The *vir* genes which can be complemented in this manner probably encode diffusible products. The number, nature, and function of these *vir* gene products remain unknown. These Ti plasmid coded functions might promote bacterial attachment to plant cells, transfer of plasmid DNA to plant cells, or integration of the T-DNA into plant nuclear DNA.

Koekman *et al.* identified the replicator region of an octopine Ti plasmid (*39*), and Ooms *et al.* isolated two transposon insertions in this region which resulted in reduced virulence (*23*); they reported difficulty in isolating Ti plasmid DNA from these mutant strains. De Greve *et*

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0036-8075/82/1126-0854$01.00/0

al. also isolated two transposon insertions in this region which reduced virulence (*21*). Avirulence may result from plasmid instability caused by transposon insertions into the replicator region.

Holsters *et al.* constructed a functional map of a nopaline Ti plasmid using insertion and deletion mutations (Fig. 2B) (*20*). They obtained 12 insertion mutants outside the T region which destroyed virulence, and two insertions near the left end of the T region which reduced the host range of the pathogen. These insertion and deletion mutants of the nopaline Ti plasmid define three separate *vir* regions outside the T region which play a role in tumorigenesis (Fig. 2B).

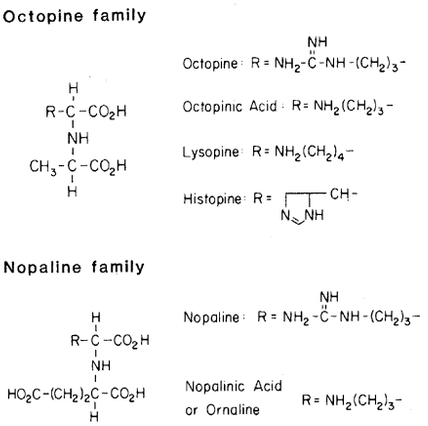


Fig. 1. Listing and partial structures of some known opines. The structures of agrocinopine family opines have not been determined.

Role of T-DNA in Tumorigenesis

Tumor formation. The effects of a large number of parameters on the process of infection of a susceptible plant by *A. tumefaciens* have been thoroughly discussed by Beiderbeck (*11*). The requirement for the entrance of the causative bacteria into a wound site is well established. There appears to be a plasmid-dependent binding of the bacteria to the plant cell wall, followed by an elaboration of cellulose fibrils by the bacteria (*40*). The direct relation between these phenomena and the actual process of tumor formation is still problematical. The details of this process probably will be more amenable to studies in which a high proportion of a cell population is synchronously transformed.

Current studies make use of tumor tissues that have been subjected to at least two powerful selective pressures: the transformed tissues must form tumors, and the tissue must be able to grow in vitro in the absence of phytohormones. The details of the processes by which the Ti plasmid or parts of it are transferred to the nucleus and specific fragments are incorporated into the host nuclear DNA remain to be ascertained. The *vir* regions referred to earlier probably play a role in the process of integration. Two possible schemes for the integration of portions of the Ti plasmid into plant DNA have been proposed by Zambryski *et al.* (*10*). The Ti plasmid may react directly with the plant DNA, or the ends of the T-DNA might first interact with one another before integration into the plant DNA.

T-DNA organization. Studies of the organization of T-DNA use cloned tumor tissues which have been subjected to the selective pressures mentioned above. In order to provide sufficient amounts of tissues from which to obtain large enough quantities of DNA, the tissues must undergo many cycles of replication. The possibility of rearrangements, deletions, or amplification of the T-DNA during this procedure should be borne in mind, as well as possible rearrangements in the plasmids used for molecular cloning of the T-DNA.

The organization of the T-DNA of a number of tumors incited by strains of *A. tumefaciens* containing octopine Ti plasmids has been studied (*41–43*). In some tumor lines, the T-DNA exists as two segments (Fig. 3). The left end of the T region (T_L) includes *tms*, *tmr*, *tml* (see below), and sometimes the octopine synthase gene (*ocs*) (*42, 43*); the right end of the T region is called T_R. Tumor maintenance requires T_L but not T_R. In nine tumor lines induced by octopine Ti plasmids, the left ends of T_L lie in Hind III fragment Y (Fig. 3), but the location of the right end of T_L varies. Ti plasmids with deletions removing the right end of the T_L indicate which areas of the T region play a role in tumor induction and maintenance. Substitution of foreign DNA for the Ti plasmid DNA lying between Eco RI fragments 7 and 13 (Fig. 3) does not affect tumorigenesis, but the resulting tumors do not synthesize octopine (*24*). Deletion of the portion of T_R lying to the right of Eco RI fragment 24 does not affect tumorigenesis and leaves *ocs* intact (*26*). Deletions removing all of T_R and part of T_L reduce virulence significantly (*26*). Usually only one copy of T_L is present per diploid cell equivalent of DNA; however, the tumor 159550/1 has at least ten copies per cell (*44*). The DNA sequence represented by T_R is found in high copy numbers in some tumors. It is not known whether cells which only contain T_R sequences are present in crown gall tumors, since these cells may not pass the selection procedure used. The occurrence of cells with T_R indicates that it should be possible to incorporate useful genes into plants in lieu of T_R.

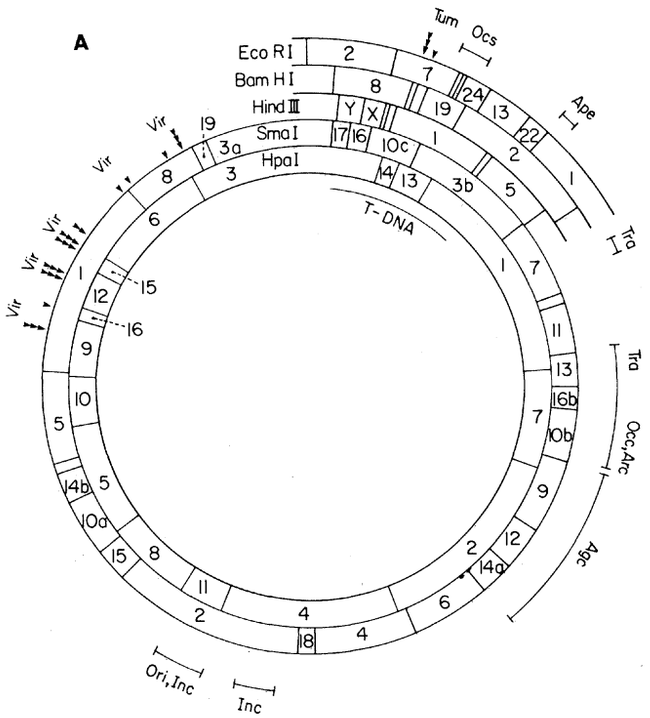
The determination of the details of the molecular architecture and the base sequence of the octopine type T-DNA has just been initiated. Studies in our laboratory have concentrated on the tumor line A6S/2 which seems to have a simple T-DNA structure. Nevertheless, the sequence determination of the first 1100 bases of the left end has revealed a number of direct and inverted repeats which are reminiscent of the sequence architecture in the adenovirus type 12 tumors (*45*). There is also a large inverted repeat present in the right end of the T-DNA (*6*). Thus, the octopine type T-DNA contains extensive regions which are precisely homologous to the Ti plasmid; however, superimposed on this regularity there is scrambling of the T-DNA sequences particularly at the junction regions between the T-DNA and the host DNA (*45*).

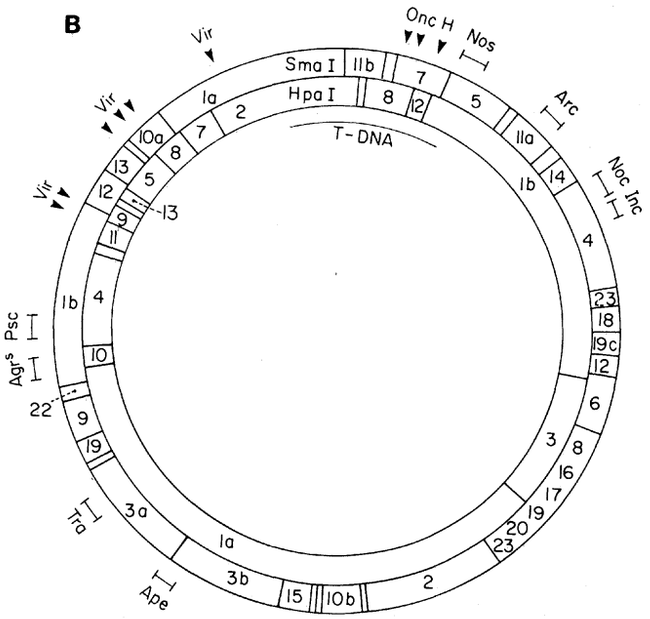
The organization of nopaline T-DNA (*10, 47*) appears to be somewhat more regular than that of octopine T-DNA. The left-hand junction can vary by about 100 base pairs while the right-hand junction appears to be exact to a base (*48*). One tumor line examined had two head-to-tail tandem repeats separated by DNA sequences of unknown origin.

Similar 23 base-pair sequences have been found near each terminus of the T region of the nopaline type Ti plasmid (*49*). An identical sequence has been found near the left terminus of the A6S/2 T region. The conservation of this sequence, if borne out by further studies, suggests that it may play a fundamental role in the insertion of the T-DNA into the host plant genome.

T-DNA Transcription and Translation in Plants

The T-DNA in tumors is transcriptionally active. A number of discrete messenger RNA (mRNA) transcripts which were homologous to the T-DNA have been detected (*50–52*). In Willmitzer's studies (*52*) the direction of transcription was determined. In Gelvin's studies (*50*) an identical pattern of transcripts was obtained from a number of octopine tumors in which the overall organization and copy number of the T-DNA were known to vary, and from tumors generat-





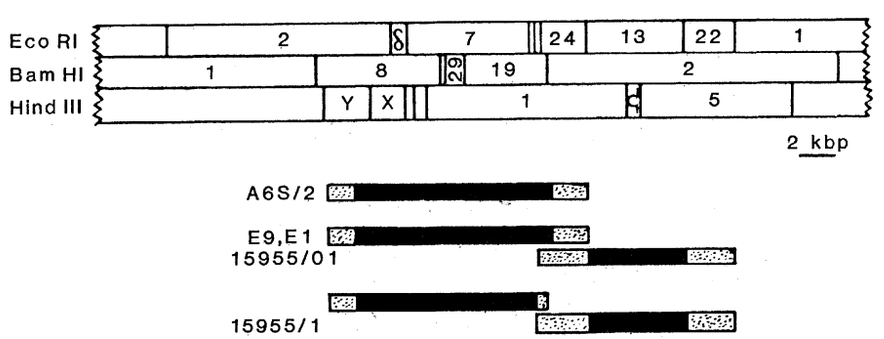


Fig. 3. T-DNA organization of several octopine type tumors. In some tumors, such as E1, E9, and 15955/1, the T-DNA occurs in two pieces termed T_L and T_R.

Fig. 2. Plasmid coded functions are as follows: Agc, agropine catabolism; Agr^s, agrocin sensitivity and agrocinopine catabolism; Ape, bacteriophage API exclusion; Arc, arginine catabolism; Inc, incompatibility; Noc, nopaline catabolism; Nos, nopaline synthase; Occ, octopine catabolism; Ocs, octopine synthase; OncH, host range; Ori, origin of replication; Psc, agrocinopine production; Tra, transfer; Tum, tumor morphology (that is, *tml*, *tmr*, *tms*); Vir, virulence. (A) Restriction endonuclease map of an octopine type plasmid (pTi-B6806). (B) Restriction endonuclease map of a nopaline type plasmid (pTi-C58). [From (*14*); courtesy of *Annual Review of Microbiology*]

ed on different hosts. This regularity in the transcription pattern of the T-DNA suggests a fundamental role of the transcripts in the maintenance of transformation of the host cells, regardless of host species. A number of the mRNA's were initiated and terminated within the T-DNA. This finding suggests that the T-DNA (although of bacterial origin) provides both promoter and terminator sequences in the eukaryotic host.

The translation products coded by T-DNA transcripts have been examined by several groups of investigators (*53–55*). In vitro translation of isolated polyadenylated mRNA molecules resulted in the formation of lysopine dehydrogenase (octopine synthase) (*53, 54*). The mRNA molecules also code for a number of other proteins of unknown function (*55*).

Crown gall tumor cells grow on chemically defined media lacking added auxins or cytokinins (*15*), and they contain higher levels of these plant growth substances than do normal cells (*12*). Normal tobacco tissue grows as an unorganized callus on tissue culture medium containing a well-defined ratio of auxins to cytokinins (*56*). Increasing the ratio of auxins to cytokinins in the medium causes root proliferation from the callus, whereas decreasing the ratio of auxins to cytokinins causes shoot development from the callus.

A clue to the manner by which the T-DNA functions to maintain the transformed state of the tumor tissue may be inferred from studies on the plant hormone levels of a number of tumors incited by wild-type or by transposon-substituted Ti plasmids (*57–59*). Strains of *A. tumefaciens* harboring wild-type Ti plasmids incite unorganized tumors. Strains that harbor octopine type Ti plasmids with transposon insertions in one of three distinct portions of the T region induce tumors with abnormal morphologies (Figs. 4 and 5), but single insertion events in the T region do not cause complete loss of virulence (*19, 21–23*). Strains that harbor Ti plasmids with insertions in a 3.1-kilobase region, called *tms* (tumor morphology shoot), cause tumors with shoots proliferating from the callus on some hosts (*19, 21–23, 60*), while other strains which harbor Ti plasmids with insertions in a 1-kilobase region, *tmr* (tumor morphology root), cause tumors with excessive root proliferation on some hosts (*19, 22–23*). Finally, strains harboring Ti plasmids with insertions in a 1.25-kilobase region, *tml* (tumor morphology large), cause tumors two to three times larger than normal on

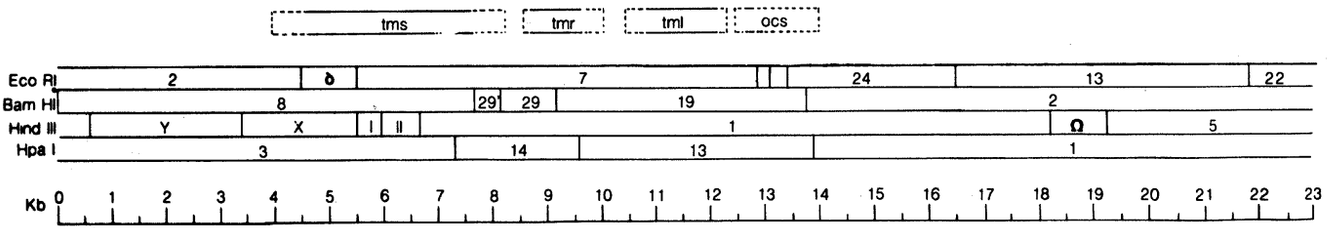


Fig. 4. Restriction endonuclease map of pTi-A6 showing the location of tumor morphology loci and the octopine synthesis locus. [Adapted from (*22*); courtesy of *Cell*]

some hosts (*22*). Tumors induced by strains carrying transposons in the *tms*, *tmr*, or *tml* regions of the Ti plasmid retain the transposon in the T-DNA (*22, 23*), suggesting that the transposon affects expression of the T-DNA in plant cells, and thereby causes an abnormal tumor morphology. Leemans *et al.* have noted a correlation between tumor morphology and some T-DNA transcripts (*61*).

Whereas a *tmr* mutation in the T-DNA of a tumor cell may increase the ratio of auxins to cytokinins in the tumor, thereby causing root proliferation, a *tms* mutation may decrease the ratio of auxins to cytokinins in the tumor, causing shoot proliferation. In support of this hypothesis, tumors incited by a *tms* strain contain roughly tenfold more *trans*-zeatin riboside (a cytokinin) than wild-type tumors, and tumors incited by a *tmr* strain contain less of this cytokinin than wild-type tumors (*59*). Also, addition of auxin to tomato plants stimulates growth of tumors incited by a *tms* strain but not by a *tmr* strain, and addition of cytokinin to tomato plants stimulates growth of tumors incited by a *tmr* strain but not by a *tms* strain (*23*). Presumably, the added growth hormones restore the normal ratio of auxin to cytokinin, permitting "normal" tumor development.

These studies suggest that the T-DNA either codes for enzymes that directly synthesize phytohormones or, alternatively, that products coded by the T-DNA affect the normal host biosynthetic or degradative mechanisms so that elevated auxin and cytokinin levels are found in crown gall tumors. The increase in phytohormones would cause the transformed tissues to grow in vitro without added growth hormones.

T-DNA Mutations Affecting Host Range

Bacteria containing nopaline type Ti plasmids induce either unorganized tumors or teratomas (unorganized tumors with abnormal shoots); the host and inoculation site determine the tumor morphology (*15*). Some insertion or deletion mutations in the T region of nopaline Ti plasmids result in reduced host range (OncH−) and some insertions also cause reduced tumor growth (Fig. 2B) (*20, 25*). Similarly, T-DNA insertions in octopine plasmids which affect tumor morphology also reduce host range (*19, 22, 23*).

Genetic Manipulation of Plants

Overall strategy. The first step in genetic manipulation of plants involves insertion of the foreign DNA of interest into a transformation vector (that is, a plasmid) capable of stably introducing this DNA into plant cells. Plant cells containing foreign genes (introduced by means of a transformation vector) are used to regenerate morphologically normal plants that carry foreign genes. Ideally, these altered plants will transmit the foreign genes through their seeds.

Regenerated plants. One of the goals of genetic engineering of plants is to introduce desired traits that will be expressed in the desired tissue, at the proper time, and preferably in such a manner that they will be transmitted through the seed. This has been achieved to a limited extent in a number of instances. Yang and Simpson generated plants from a nopaline-producing tumor by treatment with a cytokinin and showed that these plants and subsequent generations retained portions of the T-DNA present in the parental tumor (*62*). Otten and coworkers (*60*) utilized a strain of *A. tumefaciens* containing a bacterial transposon

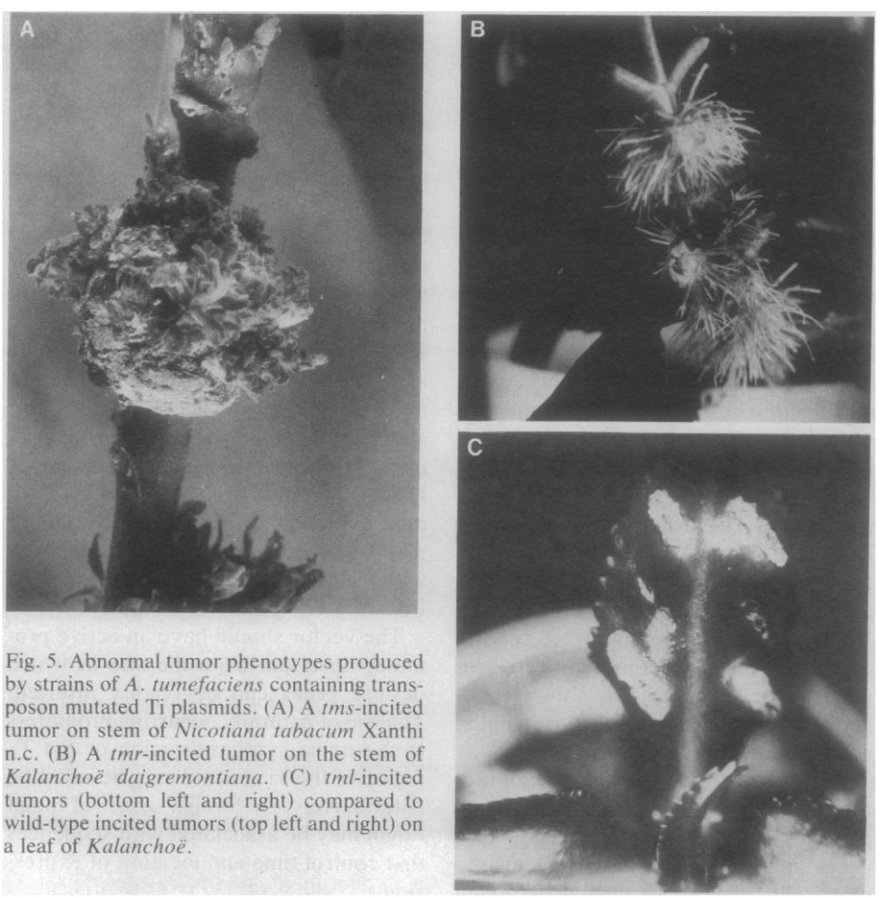


Fig. 5. Abnormal tumor phenotypes produced by strains of *A. tumefaciens* containing transposon mutated Ti plasmids. (A) A *tms*-incited tumor on stem of *Nicotiana tabacum* Xanthi n.c. (B) A *tmr*-incited tumor on the stem of *Kalanchoë daigremontiana*. (C) *tml*-incited tumors (bottom left and right) compared to wild-type incited tumors (top left and right) on a leaf of *Kalanchoë*.

in the T region of the Ti plasmid to induce tumors from which shoots grew. One of these shoots was rooted and found to contain T-DNA specific for lysopine dehydrogenase. The ability to form lysopine dehydrogenase was transmitted both paternally and maternally as a single dominant factor.

Wullems and co-workers (*63*) fused normal and tumor protoplasts and obtained callus that subsequently formed shoots. These shoots set seeds when grafted onto normal plants. In a number of cases the F_1 plants retained the ability to synthesize nopaline. More examples involving different tumors on different species are required to demonstrate the general applicability of this procedure, but the procedure appears to be promising.

A number of investigators have utilized the soil organism *Agrobacterium rhizogenes*, which incites hairy root disease (*64*). The transformed plant tissue contains plasmid-derived DNA sequences, and the tissue also synthesizes an opine similar to agropine (*29, 30, 65*). Unlike crown gall tumors, tissue transformed by *A. rhizogenes* readily forms plantlets that contain high concentrations of opines (*29, 30, 65*). This regeneration of plantlets promises to make the Ri plasmid useful for genetic engineering; however, the actual presence and organization of Ri-derived DNA in the plantlets, the morphology and stability of the regenerated plants, and the seed transmission of foreign DNA remain to be demonstrated.

Introduction of foreign DNA into the T region. Specific foreign DNA fragments have been inserted into the T region of Ti plasmids (*24, 25, 66*). Standard in vitro recombinant DNA technology was used to insert a chosen restriction fragment into a specific restriction site lying in a cloned portion of the T region. After introduction of the resulting plasmid into an *A. tumefaciens* strain harboring a wild-type Ti plasmid, homologous recombination between the two plasmids produced a Ti plasmid carrying foreign DNA in the T region. Tumors incited by an *A. tumefaciens* strain harboring such a mutant Ti plasmid contain the foreign DNA (*22–24, 67*). No confirmed reports of the expression of foreign genes in plants have yet appeared, although ongoing work indicates that this will be accomplished in the near future.

Desired traits of a transformation vector. Transformation vectors, such as Ti plasmids, are capable of carrying foreign genes and stably introducing them into plant cells. Ti plasmids that carry foreign genes in the T region stably introduce these genes into plant nuclear DNA as part of the T-DNA. The vector should be able to transform single cells or protoplasts. This has been accomplished by (i) the fusion of bacterial spheroplasts with protoplasts (*68*); (ii) the transformation of protoplasts with partially regenerated cell walls by intact bacteria (*69*); and (iii) the delivery of intact Ti plasmids into protoplasts either as free DNA in the presence of polyethylene glycol and calcium (*70*), or encapsulated in liposomes (*71, 72*). It is expected that microinjection techniques will also be used to transform single cells or protoplasts.

Methods (i) and (ii) appear to be amenable to refinement so that large numbers of single cells may be simultaneously transformed. A high efficiency of transformation would obviate the need for a selective marker. In the absence of a high degree of transformation, the vector should carry a selectable marker. A few selectable markers are now available and these promise to be useful in plant cells. A likely candidate is resistance to the antibiotic, G418, which is toxic to tobacco cells in culture (*73, 74*). The bacterial transposon, Tn*5*, confers resistance to kanamycin in prokaryotes and G418 in yeast (*75, 76*) and perhaps may function similarly in plants. Other selectable markers are being developed.

The transformed cells should not form tumors, or if they do the tumors should revert easily to morphologically normal plants retaining the foreign DNA. As was indicated earlier some crown gall tumors can be reverted to plants, and the *A. rhizogenes*–transformed tissues readily form plants. Another approach toward the development of this trait in a vector is the use of crippled Ti plasmids that do not incite tumors but nonetheless insert DNA into the plant genome. Octopine synthase activity and agropine have been detected in carrot slices infected with strains of *A. tumefaciens* harboring avirulent Ti plasmids, an indication that T-DNA integration can occur without tumor induction (*61*). The generation of plants may be more easily accomplished with the use of single cells that have been transformed by these plasmids.

The vector should have an active promoter upstream from the site in which desired genes can be readily inserted. The use of naturally occurring promoters in the opine synthesis region or the T_R region of the Ti plasmid may be suitable. Alternatively, a foreign gene of plant origin may be associated with sequences that control time and location of expression.

Conclusions

Insertion of bacterial plasmid DNA into the genome of plant cells is a naturally occurring process. In a number of instances, plants can be regenerated from transformed tissues and retain the incorporated foreign DNA. It appears that techniques for the insertion of desired genes into plants will soon be forthcoming.

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77. Supported by the National Cancer Institute grant CA13015, the American Cancer Society (IN-26V), and Standard Oil Company (Indiana); aided by grant PF-1913 from the American Cancer Society. We thank Dr. V. N. Iyer, Dr. E. W. Nester, Dr. D. J. Garfinkel, Dr. R. B. Simpson, and A. L. Montoya for critical reading of this manuscript.

Management of High-Level Waste Repository Siting

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Disposal of high-level radioactive waste is an unsolved problem of our society. Simply stated, large quantities of highly toxic liquid and solid wastes must be isolated from the environment for tens or hundreds of thousands of years. Many people may be put at risk by proximity to this waste as may their children and grandchildren. Although deep-mined geologic disposal appears to be a satisfactory means of isolating the wastes, the problem requires more than a technical solution. Our democratic principles mandate that we define a framework in which society can ratify the technical approach by approving the selection of a repository site. We suggest that the use of existing democratic processes, rather than creation of novel arrangements, is the best way to achieve social approval. The unique function of the Nuclear Regulatory Commission (NRC) seems to have been inadequately considered in the proposals made so far.

Government Roles

Federal role. The basic legal framework for high-level waste repository siting is provided by the Constitution and seven federal statutes (*1*, *2*). Although interpretations and definitions are continually evolving in the courts, the Constitution and the statutes are generally interpreted to give the federal government the right to (i) control the nuclear field; (ii) supersede state and local laws, take land, and site a repository in the name of national interests; (iii) create the authority for a repository; (iv) establish a policy of environmental protection; and (v) provide decision-making guidelines. These statutes divide the responsibility for high-level waste disposal among several federal agencies. The Department of Energy (DOE) has been designated the lead agency (*3*), the NRC has regulatory authority over DOE commercial high-level waste disposal activities (*4*), the Environmental Protection Agency (EPA) establishes radiation standards (*5*), and the Department of Transportation (DOT) is responsible for rules on the routing of radioactive materials (*6*). Lack of sufficient interagency coordination has been a key historical weakness in the national waste management program.

In his nuclear policy statement, President Reagan charged that nuclear power development has been hampered by the failure of government "in meeting its responsibility to work with industry to develop an acceptable system for commercial waste disposal." He lifted the ban that had been imposed on commercial reprocessing of high-level wastes and instructed the Secretary of Energy, "working closely with industry and state governments, to proceed swiftly toward deployment of a means of storing and disposing of commercial high-level radioactive waste" (*7*). However, no new initiatives for facilitating the process appear to be in the offing.

The legislative history of the statutes regulating nuclear power suggests that Congress intended the federal government to have absolute control over the management of radioactive wastes. Indeed, three recent court cases have seemingly affirmed federal preemption of nuclear regulation on the basis of the commerce (*8*) and supremacy (*9*) clauses

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0036-8075/82/1126-0859$01.00/0