Cell, Vol. 32, 1033–1043, April 1983, Copyright © 1983 by MIT 0092-8674/83/041033-11 $02.00/0

Regeneration of Intact Tobacco Plants Containing Full Length Copies of Genetically Engineered T-DNA, and Transmission of T-DNA to R1 Progeny

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Summary

Cloned DNA sequences encoding yeast alcohol dehydrogenase and a bacterial neomycin phosphotransferase have been inserted into the T-DNA of Agrobacterium tumefaciens plasmid pTiT37 at the "rooty" locus. Transformation of tobacco stem segments with the engineered bacterial strains produced attenuated crown gall tumors that were capable of regeneration into intact, normal tobacco plants. The yeast gene and entire transferred DNA (T-DNA) were present in the regenerated plants in multiple copies, and nopaline was found in all tissues. The plants were fertile, and seedlings resulting from self-pollination also contained intact and multiple copies of the engineered T-DNA. Expression of nopaline in the germinated seedlings derived from one regenerated plant was variable and did not correlate with the levels of T-DNA present in the seedlings. Preliminary evidence indicates that nopaline in progeny of other similarly engineered plants is more uniform. The disarming of pTiT37 by insertions at the "rooty" locus thus appears to produce a useful gene vector for higher plants.

Introduction

Ti plasmids of the plant pathogen Agrobacterium tumefaciens have the unique natural ability of transform cells of susceptible host plants by the insertion of an 8 to 23-kilobase (kb) sector of plasmid DNA into host chromosomal DNA (Chilton et al., 1977; Chilton et al., 1980; Willmitzer et al., 1980; Yadav et al., 1980; Zambryski et al., 1980). This transferred DNA (T-DNA) causes the transformed cells to synthesize new metabolites called opines (Petit et al., 1970; Bomhoff et al., 1976; Montoya et al., 1977). The synthase enzyme for one opine, octopine, has recently been shown to be encoded by a T-DNA gene (Murai and Kemp, 1982; Schröder et al., 1981). Additional genes in T-DNA affect the phytohormone balance of transformed cells as evidenced by altered morphology of tumor cells transformed by various mutant T-DNAs (Ooms et al., 1981; Garfinkel et al., 1981). Transformed plant cells containing wild type T-DNA grow in vitro without an exogenous supply of either auxin or cytokinin (Braun, 1947), whereas normal plant cells usually require both substances for growth in culture. Mutations in one T-DNA locus cause tumors from which abundant roots proliferate ("rooty" mutants), while mutations in a second T-DNA locus cause tumors from which shoots proliferate ("shooty" mutants) (Ooms et al., 1981; Garfinkel et al., 1981). Transformation of tobacco cells by shooty mutant T-DNA results in tumors which exhibit a complex auxin requirement. Such tissues will grow on hormone-free medium under conditions which allow shoot proliferation, but otherwise require auxin for continuous growth (Binns et al., 1982). The rooty and shooty functions map in a T-DNA region common to octopine, nopaline, and agropine Ti plasmids (Chilton et al., 1978; Depicker et al., 1978; Engler et al., 1981; Guyon et al., 1980), which supports the possibility that all Ti plasmids promote oncogenic growth of plant cells through a common mechanism.

T-DNA acts as a natural gene vector for A. tumefaciens, producing transformed plant cells that display an abnormal hormonal balance and synthesize new metabolites. Recent work has shown that the Ti plasmid can be exploited as an artificial gene vector to introduce novel genes into plant tumor cells (Hernalsteens et al., 1980; Leemans et al., 1982). A major obstacle to the exploitation of Ti plasmids as gene vectors for higher plants is the difficulty of regeneration of whole plants from transformed plant cell lines. Cloned teratomatous lines of tobacco cells containing wild-type nopaline plasmid pTiT37 T-DNA spontaneously regenerate shoots that displayed varying degrees of normalcy upon grafting onto healthy host plants (Braun and Wood, 1976; Turgeon et al., 1976; Binns et al., 1981). Such shoots synthesized nopaline (Wood et al., 1978), failed to form roots, and were resistant to superinfection by A. tumefaciens (Braun and Wood, 1976; Turgeon et al., 1976; Binns et al., 1981). When fertile, these grafted shoots produced seed that gave rise to apparently normal plants that lacked nopaline, produced roots, and were sensitive to A. tumefaciens (Braun and Wood, 1976; Turgeon et al., 1976; Binns et al., 1981). Indeed the cells of one such plant were shown to be completely free from T-DNA (Yang et al., 1980; Lemmers et al., 1980). Similar results have been obtained in studies of tobacco cells transformed in vitro by either octopine- or nopaline-type T-DNA (Wullems et al., 1981a; 1981b). In such experiments, opine-positive plant cells with roots were not obtained, and shoots obtained by grafting were usually both opine-positive and resistant to superinfection by A. tumefaciens. Despite male sterility in the shoots, T-DNA was shown to be passed stably through meiosis when cross-pollinated by normal plants, resulting in seedlings which contain intact T-DNA but do not develop roots (Wullems et al., 1981b). A single example has been reported of opine-positive complete plants regenerated from a crown gall tumor initially incited by a shooty mutant of octo-

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pine T-DNA (Leemans et al., 1982). These plants segregated the octopine trait in Mendelian fashion to healthy progeny, evidence that T-DNA was situated in chromosomes of the parental tissue. However, the T-DNA in these plants was found not to be full length. A large deletion of the central part of T-DNA apparently gave rise to plant cells with little T-DNA except for the octopine synthase gene (De Greve et al., 1982).

Failure of efforts of regenerate whole plants containing intact T-DNA, and isolation of plants whose T-DNA has been almost completely deleted during such efforts (Yang and Simpson. 1981; Otten et al., 1981; De Greve et al., 1982), suggest that it is necessary to disarm T-DNA by eliminating one or more genes in order to foster regeneration. Because the major obstacle to regeneration of cells containing wild-type T-DNA appears to be the inability of such cells to form roots, one might suppose that rooty mutations in T-DNA would represent a change in the correct direction. We report here that rooty mutations in the nopaline-type T-DNA of pTiT37 are indeed disarmed, at least with respect to tobacco host plants. From plant cell lines containing T-DNA genetically engineered by insertion of a model eucaryotic gene (yeast alcohol dehydrogenase I) at the rooty locus, we have obtained healthy regenerated tobacco plants containing full length T-DNA and the yeast gene in multiple copies. These plants are fertile, and their R1 progeny (seedlings derived from self-fertilization of regenerated plants; Chaleff, 1981) also contain intact multiple copies of full length engineered T-DNA. The presence of T-DNA and the expression of nopaline synthase activity in the R1 plants provides evidence of stable T-DNA transmission through gametes.

Results

Site-Specific Insertion of Yeast ADH I into the T-DNA of pTiT37

The large size of pTiT37 precludes the direct introduction of foreign DNA sequences into a precise position within the T-DNA. The gene-encoding yeast alcohol dehydrogenase I (ADH I) (Williamson et al. 1980; Bennetzen and Hall, 1982a; 1982b) was therefore introduced into a specific restriction site within the T-DNA by the indirect technique of Matzke and Chilton (1981), as shown in Figures 1 and 2. The neomycin phosphotransferase gene from Tn5, which encodes kanamycin resistance (Km^R), was attached to the yeast gene in order to allow selection for the passenger DNA during the construction. For this purpose, ColE1::Tn5 was opened at a unique Bam HI site in the transposon but outside the Km^R coding region and cloned into pBR322:ADH, a recombinant plasmid containing the 3.4-kb ADH I gene in pBR322 (Bennetzen and Hall, 1982a). Note that pBR322:ADH was opened at one of two Bam HI sites by partial endonuclease digestion. After analysis of digests of the resulting recombinant plasmids, we chose pBR322:ADH:ColE1::Tn5 which had the Km^R coding region attached directly to the yeast genomic DNA (Figure 1). Digestion of pBR322:ADH:ColE1::Tn5 with the endonuclease Eco RI, treatment with Klenow polymerase to blunt the Eco RI cohesive ends, and Hpa I digestion to remove undesired vector DNA and both termini of Tn5 produced a blunt-ended DNA fragment containing the ADH I gene and a selectable antibiotic resistance marker (Figure 1). The fragment of Tn5 remaining in this construct is incapable of transposition (Jorgensen et al., 1979).

The blunt-ended passenger DNA fragment encoding ADH I and Km^R was inserted into the same target site in T-DNA of pTiT37 chosen by Matzke and Chilton (1981), the Hpa I site in Bam HI 14a, available as a pBR325 recombinant plasmid. The construction scheme allowed direct selection for the desired product (pBR325:14a:Km^R:ADH; Figure 1), which confers Km^R from the target DNA and chloramphenicol resistance (Cm^R) from the pBR325 vector. To allow introduction of the resulting "engineered" DNA fragment into A. tumefaciens, pBR325:14a:Km^R:ADH was recloned through unique Eco RI sites into the wide host plasmid pRK290 (Ditta et al., 1980), to form pRK290:pBR325:14a:Km^R:ADH. This plasmid has the wide host range and P1 incompatibility characteristics of pRK290.

We introduced pRK290:pBR325:14a:Km^R:ADH into Agrobacterium strain A208 by transformation (Holsters et al., 1978). The resident Ti plasmid, pTiT37, contains the wild-type counterpart of the engineered Bam HI 14a fragment in pRK290:pBR325:14a:Km^R:ADH. Homologous recombination can produce cointegrates between the two plasmids; a second recombination can produce an exchange reaction (Figure 2), resulting in the transfer of the Km^R:ADH insert onto the Ti plasmid, the desired event. To select for such recombinants, we used plasmid incompatibility selection (Ruvkin and Ausubel, 1981; Matzke and Chilton, 1981), evicting pRK290 replicons with R751·pMG2, a P1 plasmid encoding gentamycin resistance (Gm^R) that can be introduced into A. tumefaciens by conjugation (Matzke and Chilton, 1981). Selection for Agrobacteria with resistance to both gentamycin and kanamycin selects for homogenotization. Four independent colonies were chosen and cloned by three successive single colony isolations on selective plates.

Ti plasmid DNA (pTiT37·ADH 1–4) was isolated from each of the four cloned strains and analyzed by Southern hybridization to determine the structure of the T-DNA. We confirmed the predicted T-DNA structure in all four strains (Figure 3) by probing Bam HI digests of the Ti plasmid DNA with pBR325:14a:Km^R:ADH. We detected only the engineered Bam HI 14a fragments and the ADH I gene. There was no visible hybridization to unaltered Bam HI 14a restric-

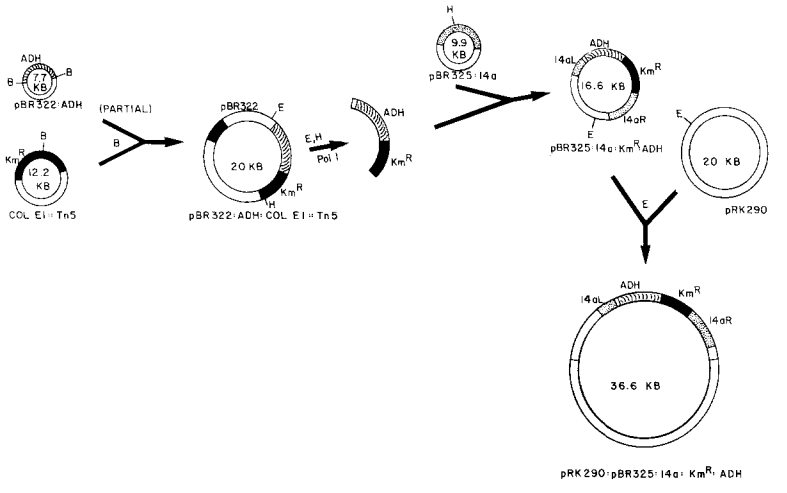

Figure 1. Construction of an Engineered Wide Host Plasmid

The engineered wide host plasmid pRK290: pBR325:14a:Km^R:ADH, with the yeast alcohol dehydrogenase I gene (ADH) and the selectable kanamycin resistance marker from Tn5 (Km^R) at the subcloned T-DNA target site, was constructed in steps requiring the sequential construction of the intermediate plasmids pBR322:ADH:ColE1:Tn5 and pBR325:14a: Km^R:ADH. pBR322:ADH is a 3.4-kb genomic clone of the yeast ADH I locus in the Bam HI site of pBR322 (Williamson et al., 1980; Bennetzen and Hall, 1982a; 1982b). Col E1::Tn5 is the plasmid Col E1 containing the active transposon Tn5, and pBR325:14a is Bam HI fragment 14a of pTiT37 cloned in pBR325 (Matzke and Chilton, 1981). The left and right sides of fragment 14a, surrounding the target site (Hpa I) are designated 14aL and 14aR, respectively. Enzymes are denoted as follows: Bam HI, B; Eco RI, E; Hpa I, H; DNA polymerase I (large fragment), Pol I.

tion fragments, nor was there hybridization to any DNA fragments of sizes representative of the free pRK290 or pBR325 vector fragments (Figure 3). We concluded that the yeast DNA sequence and attached Km^R were incorporated into the Ti plasmid by double recombination (Figure 4). All four clones of recombinant Agrobacteria were used in parallel plant transformation experiments described below.

Induction and Phenotype of Tumors from the Recombinant Ti Plasmids

For plant transformation and regeneration studies, we used isogenic A. tumefaciens strains carrying pTiT37·ADH 1–4, described above, and pTiT37·14a/a, a plasmid of similar structure containing the Hpa I subfragment of Tn5 as the passenger gene at the same T-DNA site (Matzke and Chilton, 1981). All five engineered A. tumefaciens strains incited tumors on Kalanchoë stems but not leaves, incited roots on carrot discs, and did not incite tumors on intact or decapitated tobacco plants. The positive control strain, A208, carrying wild-type pTiT37, produced tumors in all cases. To obtain transformed tobacco cells, we used an in vitro inoculation protocol (see Experimental Procedures). Tobacco cells inoculated with the engineered Agrobacteria produced nopaline and characteristically grew very slowly on hormone-free medium unless roots formed, in which case growth was considerably stimulated. Regardless of whether the cultures formed roots or not, they were extremely friable when cultured on hormone-free medium, an attribute that facilitated single cell cloning. Tissues of tumor lines HADH2 (*H*425 tobacco transformed by the pTiT37·*ADH2* bacterial strain), HADH4 and H14a/a, when shaken briefly in liquid culture medium, proved a good source of single cells. These cells were plated and grown under nonselective conditions (i.e. in medium supplemented with auxin and cytokinin) and clones were screened for nopaline. We observed considerable variation in the frequency of nopaline-positive clones obtained. From HADH2, 39 of 48 clones contained measurable levels of nopaline synthase activity, whereas only 2 of 40 from HADH4 and 2 of 45 from H14a/a, respectively, were nopaline positive.

Details of the biological characteristics of the cloned plant cell lines will be described in a separate paper. Briefly, the nopaline-positive clones exhibited the characteristics of the noncloned lines. Growth on hormone-free medium was extremely slow (Figure 5) and was accelerated by root formation, though fewer roots initiated spontaneously in the cloned lines than in the parental lines. For rapid growth of the cloned lines, it was necessary to supplement the medium with a cytokinin such as kinetin; auxin, in contrast, was not required. At high levels of kinetin (0.3 mg/l or higher), clones from HADH2 and H14a/a regularly formed buds (Figure 5), thereby mimicking the phenotype of tobacco cells transformed by the wild-type T37 strain. An important difference, however, was that buds from the HADH2- and H14a/a-transformed clones were capable of regenerating roots and growing into complete plants (Figures 5 and 6). Buds from cells transformed by wild-type T37 T-DNA form roots and must be grafted onto healthy host plants in order to reach maturity (Braun and Wood, 1976). To this date, complete plants have been derived from HADH2, HADH4, and H14a/a clones, although the HADH4 clones yield highly abnormal buds that root very poorly. All plants and buds tested to date contain nopaline, evidence of T-DNA presence in the regenerated tissue.

DNA from Regenerated Plants Contains the ADH I Gene

All plants with roots regenerated from crown gall tumors by earlier investigators have been found to

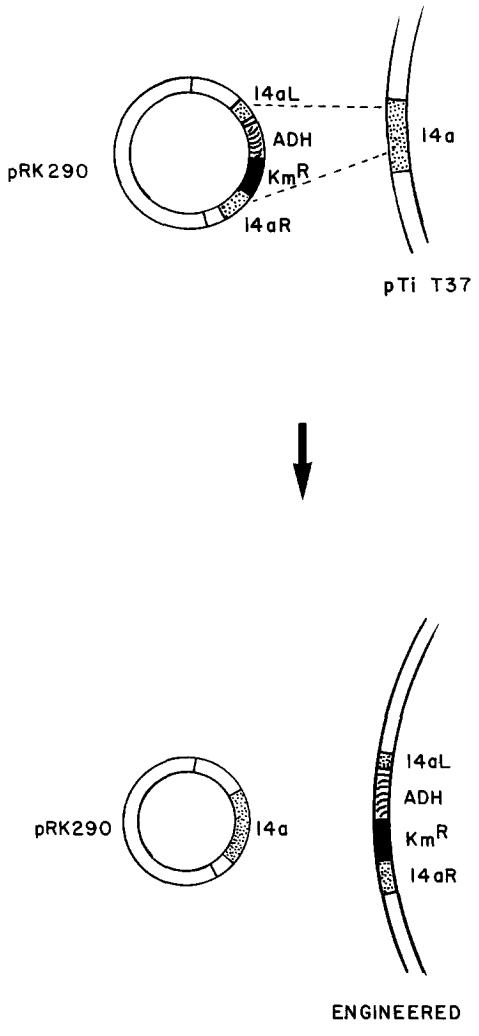

Figure 2. Exchange of the Engineered T-DNA Subfragment onto pTiT37

Recombination (– – –) occurs naturally in Agrobacterium strain A208 between homologous regions of the engineered wide host plasmid pRK290:pBR325:14aKm^R:ADH and the resident Ti plasmid pTiT37. The result of a double crossing-over is a transfer of the ADH and Km^R genes onto the Ti plasmid, with the unengineered target site being transferred to the wide host plasmid. Selection for the double crossing-over is provided by eviction of pRK290:14a from A208 using an incompatible third plasmid, and selection for the retention of Km^R (Matzke and Chilton, 1981).

exhibit large deletions covering most or all of T-DNA (Yang and Simpson, 1981; Leemans et al., 1982; De Greve et al., 1982). In our regenerated plants, the synthesis of nopaline in all tissues indicated that the right border region of T-DNA, which contains the gene-encoding nopaline synthase, was still present in the plants. In the case of the HADH2 plants, the ADH I DNA and selective marker had been inserted into T-DNA approximately 3 kb to the left of the 3′ end of the nopaline synthase transcript (Bevan and Chilton, 1982). To determine whether the yeast ADH I DNA sequence had survived in the regenerated plant, we carried out Southern hybridization analysis of DNA from transformed and normal plants using nick-translated pBR325:14a:Km^R:ADH as a hybridization probe. As shown in Figure 7, the yeast DNA and flanking Bam HI sites are present in all four plants examined. In fact, the DNA appears to be present in all clones at approximately 20 copies/genome equivalent. The probe does not show significant hybridization to normal tobacco DNA (Figure 7).

The Entire T-DNA Is Present in the Regenerated Plants

To determine whether any deletion of T-DNA had occurred, DNA from the regenerated plants was digested with endonucleases Bam HI and Eco RI for Southern analyses and and probed with a plasmid (Mini-Ti; details to be published separately) containing the entire T-DNA plus some flanking DNA of pTiT37. The probe lacks any homology with the yeast ADH insert, which therefore does not produce a band in Figure 8. A comparison of Figure 8 (A and B) with the T-DNA fragment map (Figure 4) confirms that all internal T-DNA restriction fragments are found in the DNA of plants from the four clones examined. In the analysis of Eco RI-digested plant DNAs (Figure 8A), fragments 13, 18, 23, 26, 34, and 36–38 are visible while the 1.5-kb Eco RI fragment 29 and 21-kb engineered Eco RI fragment 1 (1′), are not seen. This indicates that T-DNA borders are in Eco RI fragments 29 and 1′ as expected. The plant DNA exhibits two novel Eco RI restriction fragments of approximately 15 and 25 kb, that represent right border fragments (Lemmers et al., 1980) as determined below. The Bam HI digest of plant DNA exhibits bands of hybridization at the position of all expected internal T-DNA fragments. In addition, novel fragments are present at 2.1, 10, and 12 kb. The 2.1-kb fragment is a right border and the 10-kb fragment is a left border, as described below; we have not yet identified the 12-kb fragment.

Examination of the T-DNA Borders

We have further analyzed the DNA of the transformed plants by Southern hybridizations using T-region border clones as probes, in order to determine whether the new restriction fragments represent junctions between plasmid and plant DNA. A 3.0-kb Ti plasmid subclone (Hind III fragment 23) containing the right border, 2 kb of T-region, and 1 kb of flanking Ti plasmid DNA hybridizes to both the 15- and 25-kb novel Eco RI restriction fragments of Figure 8A (data not shown), although neither fragment is identical in size to the engineered Eco RI fragment 1 of the original transforming plasmid.

The left border of pTiT37 T-DNA is located near the right end of Eco RI fragment 29 (Figure 4), which is therefore not present as an internal fragment in the transformed plants (Figure 8A). Our failure to observe bands assignable as left border fragments in Eco RI digests of the plant DNA is expected because of the

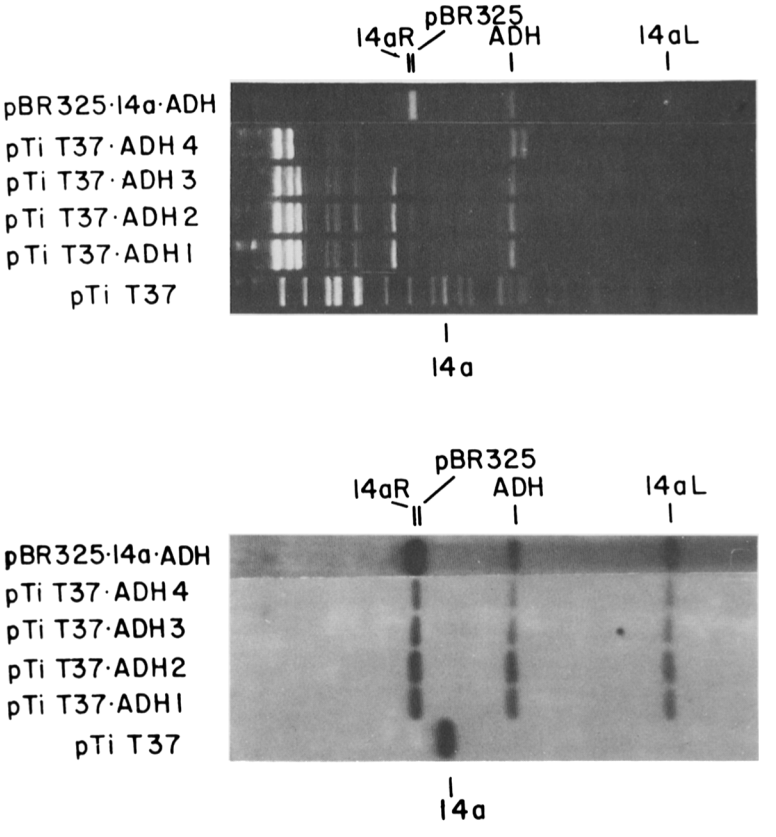

Figure 3. Confirmation of the Structure of pTiT37·ADH Plasmids

Plasmid DNA was digested with endonuclease Bam HI and electrophoresed on 0.6% agarose gels, with mobility of DNA from left to right as shown above. Top: gel stained with ethidium bromide. Bottom: autoradiogram of the same gel following Southern analysis using nick-translated DNA of pBR325:14aKm^R:ADH as a hybridization probe. The structure of pBR325.14a.ADH is detailed in Figure 1 as pBR325:14a:Km^R:ADH. pTiT37 is the resident Ti plasmid of Agrobacterium strain A208, and pTiT37·ADH1–4 are Ti plasmids from four cloned Agrobacterium strains following engineering of pTiT37 as shown in Figures 1 and 2. Densely staining bands in pTiT37·ADH1–4 of the upper gel represent restriction fragments of the evicting plasmid R751·pMG2, present in multiple copies in Agrobacterium (Matzke and Chilton, 1981). Although restriction patterns of R751·pMG2 indicate occasional rearrangements in the evicting plasmid (as in pTiT37-ADH4 above), the eviction process apparently proceeds efficiently. Southern analysis shows that the unmodified fragment 14a in pTiT37 has been replaced with the engineered fragment in pTiT37·ADH1–4.

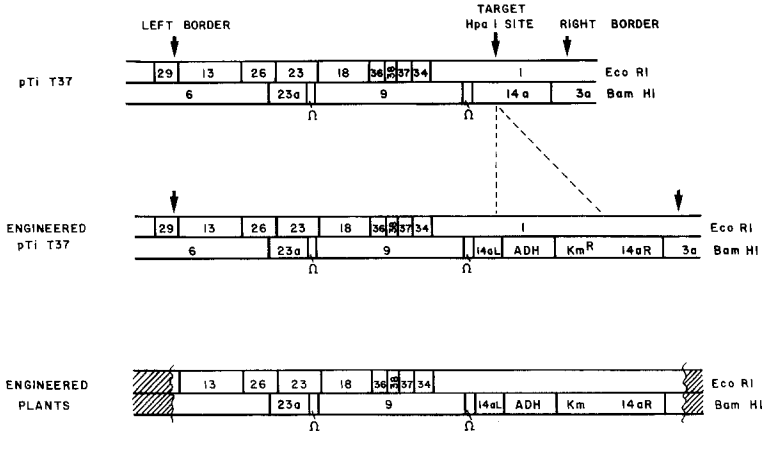

Figure 4. Restriction Fragment Maps of Original and Engineered T-DNA

Top: DNA restriction fragments found within the T-DNA of pTiT37 (region between left and right borders, approximately 22 kb in length) following digestion with endonucleases Bam HI and Eco RI are shown. The target site for insertion of the new DNA is a unique Hpa I restriction site within Bam HI fragment 14a. Middle: the restriction map of T-DNA on the engineered Ti plasmids is unchanged, with the exception of a 6.0-kb insert into the target site. Insertion of the ADH and Km^R genes into the target site provides two new Bam HI sites, resulting in the appearance of fragments 14aL, ADH, and 14aR and the loss of Bam HI fragment 14a. Bottom: the restriction map of the T-DNA in transformed plants is identical to that present on the Ti plasmid, with the exception of the border-containing fragments. T-DNA borders within Eco RI fragment 29 and Bam HI fragment 3a join with plant DNA, resulting in loss of the original restriction fragments in those regions and creation of novel T-DNA/plant DNA junction fragments (Chilton et al., 1977).

relatively short region of T-DNA (57 bp) present on the junction fragment (Yadav et al., 1982). Probing of a Bam HI digest with the Hind III fragment 23 (containing the right T-region) showed hybridization to the right-hand portion of Bam HI fragment 14a, but also to the novel 2.1-kb fragment seen in Figure 8B, which we therefore interpret as a T-DNA/plant DNA border fragment (data not shown). These findings, combined with those of the Eco RI digest described above, are consistent with the idea that the predicted right end of T-DNA has integrated into plant DNA, and that at least two new plasmid DNA/plant DNA border fragments

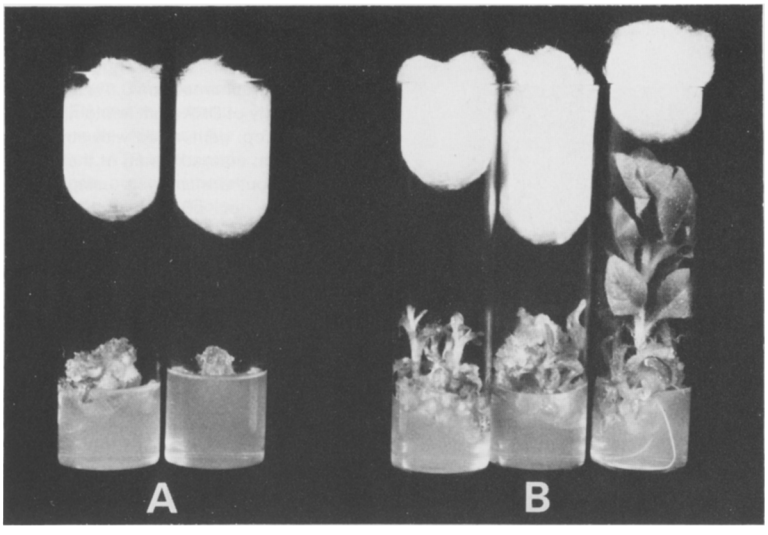

Figure 5. Growth of Transformed Tobacco Tissue on Medium with and without Cytokinin

Tobacco cells transformed with wild-type pTiT37 or engineered pTiT37·ADH Ti plasmids display varied hormonal requirements. A, growth for 4 weeks on basal MS medium (Murashige and Skoog, 1962) of pTiT37 transformed H425 tobacco cells (left) or pTiT37·ADH2 transformed cells, clone 5 (right). B, growth for 5 weeks on basal MS medium supplemented with 0.3 mg/l kinetin: (left) clone 41 of pTiT37·14a/a transformed cells; (center) clone 5 of pTiT37·ADH2 transformed tobacco cells; (right) clone 24 of pTiT37·ADH2 transformed cells.

Figure 6. Regenerated Plants and Progeny from Transformed Tobacco Cells

Left to right: Havana 425 tobacco grown from seed, 11 weeks old; plant 25 regenerated from HADH2 clone 24 cells, 10 weeks; nopaline-positive progeny grown from seed of self-pollinated HADH2 clone 24 plant 2, 12 weeks; nopaline-negative progeny grown from seed of HADH2 clone 24 plant 2, 12 weeks.

have resulted. Further analysis is required to explain the difference in number of unique right border sequences between the two digests.

Probing of Bam HI-digested DNA with a left border-containing Ti plasmid subclone (Bam HI fragment 6) revealed a single fragment of 10 kb, slightly larger than the 9.5-kb Bam HI fragment 6. No hybridization was seen to the 12-kb Bam HI fragment, which we cannot yet identify. This result suggests the presence of a unique left T-DNA border. Because only 40% of a junction fragment of this size should be homologous to the hybridization probe, the relative intensities of bands in Figure 8B suggest that the border fragment is present in the plant genome in approximately as many copies as the internal T-DNA fragments. We cannot yet explain the high copy number of left and right border fragments, and have found no evidence that a tandem insertion has occurred, fusing left and right T-DNA borders (Lemmers et al., 1980).

R1 Seedlings Contain Nopaline

Plants derived from clones HADH2 and H14a/a were allowed to flower, were self-pollinated, and set seed. Nopaline assays of the germinated R1 seedlings from one plant of HADH2-cloned cells showed that 46 of 200 seedlings examined contain nopaline, with relative levels of nopaline varying greatly among the nopaline-positive plants. We are currently examining the progeny of many additional plants, derived from nine different HADH2 clones and two H14a/a clones. Preliminary results show that a higher proportion of these seedlings contains nopaline.

The T-DNA Is Present in Germinated R1 Seedlings

The presence of nopaline in some of the R1 progeny indicated that at least a portion of T-DNA had survived meiosis. To determine whether deletions had occurred in the T-DNA of the progeny, we carried out Southern analysis of DNA isolated from young seedlings con-

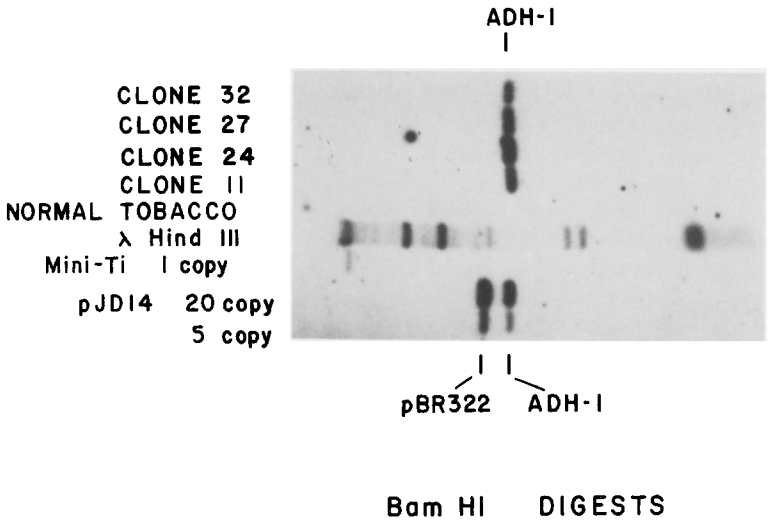

Figure 7. Regenerated Plants Contain the Yeast ADH I Gene

DNA was isolated from normal Havana 425 tobacco, and from plants regenerated from cloned cultures 11, 24, 27, and 32 of tobacco transformed with engineered pTiT37·ADH2. The DNA was digested with endonuclease Bam HI, electrophoresed on 0.6% agarose gels, and transferred to nitrocellulose for Southern analysis. The hybridization probe used was nick-translated pBR322:ADH, a clone of yeast ADH I in pBR322 (Bennetzen and Hall, 1982a). Mini-T, a clone of T-DNA with partial homology to pBR322 (A. de Framond, personal communication), was electrophoresed at a concentration corresponding to 1 copy/genome equivalent. pJD14 (pBR322:ADH, Bennetzen and Hall, 1982a; 1982b) is present at 5 copies and 20 copies/genome equivalent. Mobility of DNA on gels was left to right as shown above.

taining a high level, medium level, or zero nopaline. Analysis of Eco RI digests (Figure 9) or Bam HI digests (data not shown) confirmed that all contain complete and multiple copies of the original transforming T-DNA, including multiple copies of the nopaline synthase gene. The copy number of the T-DNA appears to be similar in the three different seedlings, and does not appear to correlate with the level of nopaline production. R1 seedling 14, which contains no nopaline synthase activity, has an additional hybridizing DNA fragment of smaller size and lower copy number than the two right border-containing fragments (Figure 9), but we cannot say whether this rearrangement is related to levels of nopaline synthase expression. We intend to extend this study to additional seedlings derived from the same parent, as well as to seedlings derived from alternative HADH2 and H14a/a plants.

Discussion

Regeneration of Plants from Tobacco Cells Transformed by Rooty Mutants of pTiT37

The natural ability of Agrobacterium Ti plasmids to transform plant cells by inserting new DNA into the host plant genome makes these plasmids an obvious point of departure for development of practical plant transformation vectors. However, the oncogenic transformation that T-DNA confers on plant cells has been found to prevent their regeneration to intact, healthy plants, an essential step for useful exploitation of this vector system. Mutations engineered into the Hpa I site of pTiT37 fragment 14a (Matzke and Chilton, 1981; this work) affect morphology of the transformed plant cells, causing them to be prone to root initiation. The "rooty" mutations in octopine T-DNA mapped by Ooms et al. (1980) and Garfinkel et al., (1981) are located in the part of T-DNA common to octopine and nopaline Ti plasmids, in a region encoding a 1.2-kb transcript (Bevan and Chilton, 1982; Willmitzer et al., 1982; Gelvin et al., 1982). The Hpa I site in pTiT37 Bam HI fragment 14a falls within the region encoding that transcript (Bevan and Chilton, 1982). By directing the insertion of foreign DNA into this site, which is in the rooty locus on nopaline-type T-DNA, we have been able to overcome the problem of regeneration of plants from transformed cells. Results presented here demonstrate that engineered Ti plasmids with such a mutation induce attenuated crown gall tumors that are not cytokinin autotrophic as are wild-type T37-induced tumors. Moreover, the cloned transformed tobacco cells containing such mutant T-DNA are prone to both root initiation and regeneration of intact plants.

The plants regenerated from both HADH2 and H14a/a transformed cell lines appear quite normal, with only minor morphological differences from wild-type plants, such as slightly elongated stigmas. While it is possible this results from the presence of T-DNA in the cells, such differences may also be due to passage of the cells through tissue culture. The presence of nopaline in all tissues of the regenerated plants indicates that at least one T-DNA gene, that encoding nopaline synthase, remains functional. Preliminary data indicate that when tissues from regenerated plants are returned to culture they exhibit the auxin-independent, cytokinin-dependent phenotype of the original clones (A. Binns, unpublished data). It therefore appears that plant cells are capable of either regulating T-DNA genes or modulating auxin levels efficiently, allowing the growth and development of normal plants. A transcriptional study of T-DNA genes in the regenerated plants will prove valuable in determining the extent of expression of the shooty locus and other remaining T-DNA genes following regeneration of plants.

Passage of Full Length T-DNA through Gametes to R1 Progeny

Previous reports have indicated that fragments of T-DNA can be transmitted to normal R1 seedlings

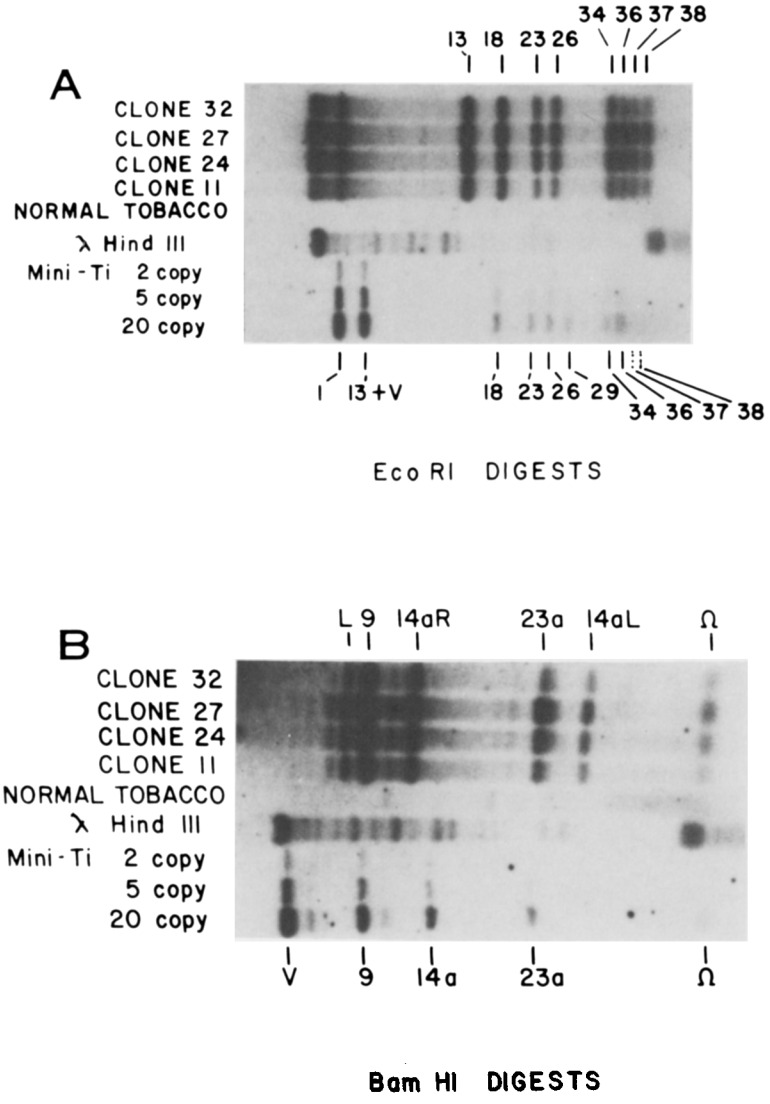

Figure 8. Regenerated Plants Contain Full Length T-DNA

DNA isolated from normal and engineered tobacco plants was digested with endonucleases Eco RI (A) or Bam HI (B) and electrophoresed on 0.6% agarose gels. Southern analyses were carried out using nick-translated Mini-T as a hybridization probe. Hind III-digested λ DNA is included as size marker, with electrophoretic mobility from left to right. Bam HI-digested Mini-T is present at 2, 5, and 20 copies/genome equivalent. Fragment numbers (top and bottom) correspond to those of Figure 4.

through meiosis only if deletions have occurred that eliminate the transforming genes (Yang and Simpson, 1981; Otten et al., 1981; De Greve et al., 1982). The plants regenerated in this study transmit full length T-DNA to their normal R1 progeny.

Expression of Nopaline Synthase in R1 Progeny

It is not clear from our present data whether the multiple copies of T-DNA in parental tissues are genetically linked, so we cannot predict what frequency of T-DNA transmission to R1 progeny is expected. If all copies of T-DNA were integrated at a single genetic locus, 75% of the progeny from self-pollination would be expected to contain multiple copies of T-DNA. If T-DNA is inserted at multiple sites, the transmission rate would be still higher with a distribution of copy numbers. Our finding that nopaline was present in only 24% of the progeny was therefore unexpected. Analysis of octopine synthase transmission through seed (Otten et al., 1981) indicated classical Mendelian segregation of that gene as a dominant trait. We note that the plant of Otten et al. had a single copy T-DNA insert, in contrast to our multicopy T-DNAs. Although we have now analyzed by Southern hybridizations the DNA of only three R1 seedlings derived from one HADH2 parent (regenerated from clone 24), it is already apparent that some seedlings contain multiple copies of T-DNA but do not express nopaline synthase activity. All of the seedlings examined contained approximately equal doses of T-DNA, yet nopaline levels varied from high level to none. This puzzling observation finds a parallel in mouse genetic engineering studies recently reported by Palmiter et al. (1982): the level of expression of metallothionein-thymidine kinase fusion genes in offspring of transgenic mice does not correlate well with copy number of the gene, and expression of the gene in offspring may be absent, reduced, or enhanced, relative to the level of expression in the parent. Our preliminary studies of R1 seedlings derived from other HADH2 and H14a/a

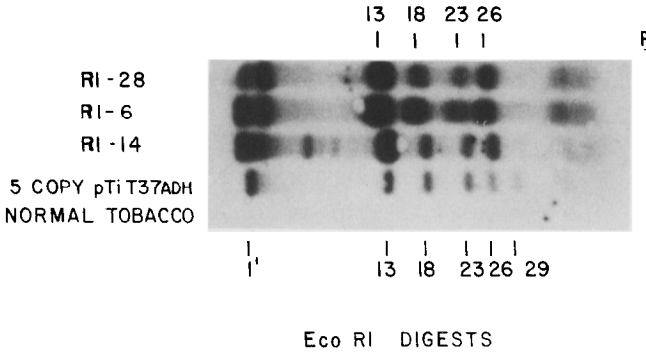

Figure 9. T-DNA Is Present in R1 Progeny of Engineered Plants

Regenerated plant 2 from HADH2 clone 24 was self-pollinated, and germinated R1 seedlings were analyzed for presence of nopaline. Eco RI-digested DNAs isolated from seedlings containing a high level (R1-28), a medium level (R1-6), and no detectable nopaline (R1-14) were analyzed by Southern hybridization using nick-translated Mini-T as probe. Eco RI-digested DNA of pTiT37-ADH1 is present at 5 copies/genome equivalent for size and copy number reference; fragment numbers (top and bottom) correspond to those of Figure 4.

plants demonstrates a more stable phenotypic expression of the nopaline synthase activity following meiosis, and is under further investigation.

The Yeast ADH I Gene Does Not Express in Tobacco

Experimental results relating to expression of the yeast ADH I gene have not been included in this paper. Thus far, we have been unable to detect yeast ADH protein immunologically through western blots or immunoprecipitation (Sixma et al., 1982) or enzymatically (Torres, 1974), either in tissue culture or in regenerated plants. We have also found no evidence of polyadenylated transcripts homologous to the yeast ADH I gene in regenerated plant tissues under conditions in which the nearby T-DNA transcripts are detected (Bevan and Chilton, 1982). The apparent lack of expression is disappointing but not surprising. The potential regulatory DNA sequences in the ADH I gene are representative of higher eukaryotic genes (Bennetzen and Hall, 1982a; 1982b). However, precise requirements for expression of DNA sequences at various developmental stages in plants are not known. The general approach used here to insert the ADH I DNA into the genome of intact plants should afford access to such information.

Experimental Procedures

Materials

We obtained α-^{32}P-dCTP (specific activity > 400 Ci/mmol) from Amersham Corp. Restriction endonucleases, DNA polymerase (Klenow fragment), and T_4 DNA ligase were obtained from New England Biolabs and Bethesda Research Laboratories, and were used according to the suppliers' directions. All antibiotics were purchased from Sigma.

Bacterial Strains and Plasmids

The genomic clone of yeast alcohol dehydrogenase I in pBR322 (pJD14) was provided by J. Bennetzen (Bennetzen and Hall, 1982a; 1982b). A. tumefaciens strain A208 is a transconjugant containing Ti plasmid pTiT37 in the cured strain C58 genetic background (Sciaky et al., 1978). The plasmid ColE1::Tn5 was obtained from D. Berg. pBR325(Bam HI 14a) is a recombinant plasmid containing the T-DNA fragment Bam HI fragment 14a of pTiT37 (Yadav et al., 1980). Wide host range plasmid pRK290, provided by D. Helinski, is in the P1 incompatibility group, and is a nonconjugative but mobilizable derivative of RK2 (Ditta et al., 1980). R751-pMG2, a recombinant derivative of R751 and pMG2 and also of the P1 incompatibility group, was supplied by G. Jacoby. The plasmid pTiT37-14a/a, constructed by Matzke and Chilton (1981), is an insertional mutant of pTiT37.

Bacterial Growth Media

L-broth (1% tryptone, 1% NaCl, 0.5% yeast extract) and YEP broth (1% peptone, 1% yeast extract, 0.5% NaCl) were used for liquid cultures of E. coli and A. tumefaciens, respectively. E. coli strains were plated on L-broth containing 1.5% agar and appropriate antibiotics. A. tumefaciens strains were plated on nutrient agar (Difco) supplemented with appropriate antibiotics.

Plasmid DNA Isolation

E. coli plasmids were isolated and purified on CsCl/ethidium bromide density gradients as reported previously (Matzke and Chilton, 1981). Ti plasmid was prepared by the method of Currier and Nester (1976), except that shearing of the lysate was not carried out (Matzke and Chilton, 1981).

Conjugations and Transformations of Bacterial Cells

Transformations of E. coli and Agrobacterium were carried out as previously described (Matzke and Chilton, 1981). Conjugation between E. coli and A. tumefaciens was accomplished by combining 0.2 ml of L-broth and 0.2 ml each of rapidly growing E. coli (strain HB101 containing gentamycin-resistant R751-pMG2) and A. tumefaciens (containing pTiT37 and the kanamycin-resistant engineered wide host plasmid). After 2-4-hr incubation without agitation at 30°C, cells were plated on nutrient agar supplemented with kanamycin (100 μg/ml) and gentamycin (50 μg/ml), and incubated at 30°C. Individual colonies appeared in approximately 48 hr and were picked and cloned by three successive single colony isolations on plates containing kanamycin and gentamycin.

Inoculation of Tobacco Stems by Recombinant Bacteria

A. tumefaciens strains with insertional mutations at the Hpa I restriction site of fragment Bam HI 14a did not induce tumors on tobacco when inoculated by needle puncture into intact plants. For isolation of transformed tobacco cells, we used the technique developed by Braun (1956) for transformation of stem segments in vitro. Stems of N. tabacum var. Havana 425 were surface sterilized with 7% commercial Chlorox and 80% ethanol, rinsed with sterile distilled water, and cut into 1-cm long segments. These were placed basal end up in Petri dishes containing Murashige and Skoog medium (Murashige and Skoog, 1962) (MS medium) without hormonal supplement. The basal end was then inoculated with bacteria, puncturing the cut surface of the stem by syringe needle. After 5-8 days of incubation at 25°C with 16-hr light, callus developed at the upper surface of all stem segments including those inoculated with avirulent strain A136. The callus regions were then transferred to hormone-free MS medium containing carbenicillin (200 μg/ml). After three transfers at 4-week intervals on this medium, the tissues were free of bacteria and could be assayed for growth and nopaline content.

Plant Tissue Culture and Cloning

Once free of inciting bacteria, plant tissues were grown on MS medium at 25°C with 16-hr light and 8-hr dark. These tissues were

cloned using a suspension method described previously (Binns and Meins, 1979). Briefly, tissues were placed in liquid MS medium supplemented with 0.02 mg/l naphthalene acetic acid (NAA) and shaken at 135 rpm and 25°C for 2–3 days. The resultant suspensions were filtered successively through 543- and 213-μm stainless steel mesh, concentrated, and plated at a final density of 8×10^3 cells/ml in 5 ml of MS medium containing 0.5% agar, 2.0 mg/l NAA, 0.3 mg/l kinetin, and 400 mg/l yeast extract (Difco). Good suspensions of individual cells were obtained from the tumors transformed by recombinant bacterial strains and routinely gave 30–50% plating efficiency. Once colonies had reached 1 mm in diameter, they were picked by scalpel point and placed on complete MS medium (i.e. supplemented with 2.0 mg/l NAA and 0.3 mg/l kinetin). After these had grown to approximately 50 mg, colonies were split into three pieces. One was placed on hormone-free MS medium, and one on complete MS medium, and the third piece was placed on MS supplemented with 5 mM arginine to be used for nopaline analysis (Otten and Schilperoort, 1978).

Regeneration of Recombinant Plants

Tissues from various nopaline-positive clones were transferred onto MS medium supplemented with 0.3 mg/l kinetin, and cultured at 25°C with 16-hr light and 8-hr dark. Shoots initiated were subsequently rooted by placing them in a medium consisting of 1/10 strength MS salts, no sucrose or hormones, 0.4 mg/l thiamine, and 1.0% agar, with the pH adjusted to 7.0. Rooted plantlets were transferred to soil and placed at high humidity in a greenhouse. After 7–10 days, the plants were then grown with normal greenhouse conditions.

Plant DNA Isolation

High molecular weight DNA was isolated as described (Chilton et al., 1982) from young tobacco plants frozen in liquid nitrogen and ground in a mortar with pestle. Following banding in CsCl-ethidium bromide gradients, dye was extracted with isopropyl alcohol equilibrated with 20× SSC (3 M NaCl, 0.3 M sodium citrate), an equal volume of 0.6 M sodium acetate was added, and DNA was precipitated by the addition of absolute ethanol to a final concentration of 70%.

Southern Hybridization Analysis of DNA

Plant or plasmid DNA was digested to completion with restriction endonucleases and loaded into 1 × 8 × 8-mm wells in a horizontal 0.65% agarose gel prepared in Tris-acetate buffer (Chilton et al., 1977). Electrophoresis, transfer of DNA to nitrocellulose, and Southern hybridizations were carried out as described by Thomashow et al. (1980). For analysis of Ti plasmid structure in Agrobacterium, a total of 2 μg of plasmid DNA was loaded per gel track, with standard plasmid digests run on the same gel at 0.1 μg/track. For plant genomic analyses, plant DNA was loaded at 10 μg/gel track; standard plasmid digests corresponded to the indicated genome equivalents (Chilton et al., 1977). All standard plasmid digests on plant genomic DNA gels were digested with endonucleases and electrophoresed in the presence of 10 μg of calf thymus DNA/track as carrier. Hybridization probes were generated by nick translation of purified plasmid DNA, to a specific activity of approximately 10^8 cpm/μg of DNA.

Acknowledgments

The authors wish to thank Dr. Jeffery Bennetzen for providing the yeast alcohol dehydrogenase genomic clone and for sharing pertinent DNA sequence information prior to publication, and Sara Piddington for expert technical assistance. This work was supported by American Cancer Society and Department of Energy grants to M. D. C., a National Science Foundation grant to A. N. B., Monsanto Fellowship Funds to K. A. B. and A. J. M. M., and a National Research Service Award to K. A. B.

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Received November 1, 1982; revised January 27, 1983

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